“Seedborne microbiota from tolerant rice genotype: a new approach to enhance salt-tolerance in sensitive varieties”

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Resumo

O arroz é uma cultura essencial mundialmente, mas é altamente suscetível a condições de stresse abiótico, incluindo a salinidade do solo, que é um grande problema para a agricultura. As bactérias promotoras do crescimento de plantas (BPCP) podem promover o vigor e a adaptação das plantas em condições de stresse, produzindo hormonas de crescimento e outros compostos. A identificação de BPCP tolerante ao sal pode levar a novos mecanismos para lidar com o stresse salino e fornecer soluções sustentáveis para a agricultura. O nosso projeto teve como objetivo estudar o efeito de candidatos BPCP, isolados da semente e endosfera de plantas de arroz tolerantes ao sal (Pokkali) e o seu impacto ao nível de resistência ao stresse salino e as respostas das variedades sensíveis ao sal (Nipponbare e IR -29). Estávamos particularmente interessados nas sementes, porque a planta-mãe transfere bactérias especificas à próxima geração, com a intenção de aumentar a sua capacidade de adaptação. Inicialmente, o microbiota cultivável proveniente da zona endofitita de raízes e sementes de todas as cultivares foi isolado e identificado geneticamente para avaliar os padrões populacionais. Em seguida, um total de oito cepas foram selecionadas e avaliadas pelas suas características e taxa de crescimento em sal em diferentes concentrações (até 2 M), na formação de biofilme, auxinas e produção de ACC desaminase. Quatro das oito cepas testadas foram selecionadas (*Bacillus altitudinis, Bacillus subtilis, Lisinibacillus fusiformis* and *Paenibacillus pabuli*). Os efeitos destas cepas no desenvolvimento fisiológico da cultivar sensível ao sal Nipponbare foram testados em condições hidropónicas não salinas e salinas, onde três das quatro cepas (*Bacillus altitudinis, Bacillus subtilis* and *Paenibacillus pabuli*) revelaram tendências positivas em relação à melhora da tolerância ao sal. Além disso, decidimos fazer uma primeira abordagem para estudar o nível de metilação do DNA das plantas quando inoculadas com as cepas candidatas e submetidas a condições de salinidade. Esta análise foi realizada com a técnica Chop-PCR e teve como alvo oito genes. Os resultados mostraram-se promissores em relação aos genes OsHKT2;1 e OsCATA, sendo estes relacionados com o transporte de membranas e ao combate dos efeitos aversivos de Espécies Reativas de Oxigênio. Esses candidatos mostram potencial para serem usados como um bio-inoculante na melhora do desempenho do arroz sob condições de stresse salino.

Palavras- chave: Salinidade, Arroz, Endofiticos, BPCPs, Marcas epigenéticas

Abstract

Rice is an essential crop worldwide, but it is highly susceptible to abiotic stress conditions, including soil salinity, which is a major problem for agriculture. Plant growth-promoting bacteria (PGPB) can promote plant vigour and adaptation in stressful conditions by producing phytohormones and other compounds. Identifying salt-tolerant PGPB can lead to novel mechanisms for coping with salt stress and providing sustainable solutions for agriculture. Our work aimed to study the effect of ST-PGPB candidates isolated from the seed-borne and endosphere of salt-tolerant rice plants (Pokkali) and their impact on the level of salt stress resistance and responses of the salt-sensitive varieties (Nipponbare and IR-29). We were particularly interested in the seed-borne because the mother plants transfer the bacteria to the next generation in order to provide better adaptability. Initially, the culturable seed-borne and root endophytic microbiota of all cultivars was isolated and genetically identified to evaluate population patterns. Then, a total of eight strains were selected and screened by their skills and growth rate in salt, at different concentrations (up to 2 M), including biofilm formation, auxins and ACC deaminase production. Four of the eight tested strains were selected (*Bacillus altitudinis, Bacillus subtilis, Lisinibacillus fusiformis* and *Paenibacillus pabuli*). The effects of these strains on the physiological development of the salt-sensitive cultivar Nipponbare were tested under non-saline and saline hydroponic conditions, where three of the four strains (*Bacillus altitudinis, Bacillus subtilis* and *Paenibacillus pabuli*) reveal positive tendencies regarding salt-tolerance improvement. Furthermore, we decided to take a first approach to study the DNA methylation level of the plants when inoculated with the candidate strains and subjected to salinity conditions. This analysis was performed with Chop-PCR technique and targeted eight genes. The results showed to be promising regarding the genes OsHKT2;1 and OsCATA, being these related to membrane transport and the combat of aversive effects of ROS. These candidates show a potential to be used as a bioinoculant treatment to amend rice performance under saline-stress conditions.

Keywords: Salinity, Rice, Endophytes, ST-PGPB, Epigenetic trait

Table of Contents

List of Figures

Abbreviations and Symbols

ACC – 1-aminocyclopropane-1-carboxylic acid

ACC deaminase – 1-aminocyclopropane-1-carboxylic acid deaminase

BLAST – Basic Local Alignment Search Tool

Ci – Initial concentration

Cf – Final concentration

CFUs – Colony Forming Units

ddH2O – Double distilled water

DW – Dry Weight

E - Evenness

EC - Electrical conductivity

EPSs – extracellular polymeric substances

GB – glycine betaine

H - Shannon diversity index

IAA – Indole-3-acetic acid

LB – Luria Bertani

MM9 – M9 minimal media

MSRE – methyl-sensitive restriction enzymes

NIP - Nipponbare

OD – Optic Density

P – phosphorus

PCR – Polymerase Chain Reaction

PGP - Plant Growth Promoting

PGPB - Plant Growth Promoting Bacteria

POK - Pokkali

ROS – Reactive Oxygen Species

ST-PGPB – Salt Tolerant - Plant Growth Promoting Bacteria

Unk. – Unknown (unidentified)

Vi – Initial volume

Vf – Final volume

Introduction

Contextualization

Rapid population growth in the twenty-first century has been accompanied by severe problems in the agroecosystems of the world, resulting in lower production and the degeneration of sustainable agriculture (Mohanty *et al.*, 2021). Moreover, the world's population is expected to reach 9.7 billion by the end of 2050, and food demand is projected to increase by 85% (FAO, 2017). Rice, as a staple crop, is a crucial source of nutrition and a means of subsistence for billions of people globally. For more than half of the world's population, it serves as their primary source of food (Fukagawa and Ziska, 2019). Both rice production and consumption have risen throughout time in response to the expanding global population. As of 2019, there were 501 megatons of milled rice produced globally, with an average daily consumption of 179 grams per person, a considerable rise of 19.3% since 2000 (FAO *et al.*, 2020). Notwithstanding this rise, rice-producing nations confront obstacles like population expansion, a decline in rice-planting land, and global climate change, all of which affect rice output (Tang *et al.*, 2022).

Soil salinity is a significant constraint to crop productivity worldwide, and it is particularly detrimental to rice production, especially in regions where irrigation water is obtained from saline water sources (Yang *et al.*, 2022). The ability of rice plants to absorb water and nutrients is inhibited by excessive salt in the soil, which results in stunted growth, lower yields, and even crop failure (Hussain *et al.*, 2017). Additionally, to the decrease in soil fertility, salt build-up in the soil over time can negatively impact the ecosystem. Moreover, excessive salt content in irrigation water can cause soil erosion and land degradation, which reduces the quantity of land suitable for rice farming (Singh, 2021). According to (FAO, 2021) over 1,100 million hectares of soils are affected by salinity and sodicity, of which 60% are saline, 26% sodic and the remaining 14% are saline-sodic. Salt-affected soils are found on all continents, with the most affected regions being the Middle East, Australia, North Africa, and Eurasia. There are also many parts of Europe highly affected by soil salinity, but the Mediterranean basin and the eastern part of the continent are the most affected (Hassani, Azapagic and Shokri, 2021).

Rice farming is a significant contributor to greenhouse gas emissions and environmental harm, mainly through the excessive use of water and fertilizers. Therefore, sustainable rice production technologies are needed to increase rice production while reducing its environmental impact (Mboyerwa *et al.*, 2022). To address this issue, effective management measures are required, and one potential solution is the use of plant growth-promoting bacteria (PGPB). These bacteria have been found to mitigate the negative effects of soil salinity by improving rice plant nutrition, balancing hormones, and producing osmoprotectants and antioxidants (Kumar *et al.*, 2020; Shultana *et al.*, 2022). Environmental factors, such as salt stress, have an impact on the plant’s mechanisms, leading to epigenetic modifications. DNA methylation is a well-known epigenetic modification, that affects gene expression and plant growth (Miryeganeh, 2021). PGPB can mitigate the negative effects of salt stress by modulating DNA methylation patterns, leading to increased growth and stress resistance (Chen *et al.*, 2022).

In conclusion, the sustainability of rice production is critical for global food security, as the world's population is projected to continue growing, and demand for food will increase accordingly. However, rice farming faces several challenges, one of which is soil salinity, which negatively affects crop productivity and the environment. The use of PGPB offers a promising solution to mitigate the negative effects of salt stress and improve rice plant growth, as these bacteria can promote plant growth by improving nutrient uptake and inducing systemic resistance against pathogens. Additionally, epigenetic modifications, such as DNA methylation, can provide valuable insights into developing crop varieties that are more tolerant to salt stress. Through the identification and manipulation of salt-tolerant genes, it is possible to create crop varieties that can withstand harsh soil conditions and produce high yields. To ensure sustainable agriculture, effective management measures must be implemented, such as using precision farming techniques to optimize crop yields while minimizing environmental impacts. In this way, we can increase rice production to meet the growing demand for food while also minimizing the negative effects on the environment. Furthermore, sustainable rice production can help reduce poverty in many rural areas, as it is an essential source of income and food for millions of small-scale farmers worldwide. By addressing the challenges faced by rice farming and promoting sustainable agriculture practices, we can help build a more resilient and food-secure future for all.

The Impacts of Soil Salinity on Plant Growth and Agriculture: Causes and Effects

The presence of fertile agricultural soil with all necessary environmental and nutritional conditions ensures successful plant growth and development, resulting in consistent, high-quality yields. However, saline soils contain elevated levels of soluble salts that impede plant growth by causing several adverse effects (Shilev, 2020). Saline soils are defined as soils with an electrical conductivity exceeding 4 dS/m at 25°C (Richards, 1954). There are two ways salinization can occur. The first is through natural or primary means, where soils have an innate characteristic of high salt content, such as in salt lakes, scalds formation, seawater influence, and the release of soluble salts from rocks and minerals. The second is through artificial or secondary means, which is caused by human activities like chemical contamination, improper crop rotations, over-irrigation, flooding, and inadequate drainage (Hoang *et al.*, 2016; Mohanavelu, Naganna and Al-Ansari, 2021).

Saline stress is considered a severe challenge affecting both biotic and abiotic environments, especially as it is closely linked to water and exacerbated by climate change. In arid and semi-arid regions, the problem is particularly widespread and problematic, and it can be further compounded by drought conditions (Shrivastava and Kumar, 2015). Soil salinity significantly reduces the amount of cultivated land by converting it into uncultivated land at an estimated rate of 1% to 2% each year, particularly in arid and semi-arid areas (FAO, 2021). In Spain, approximately 3% of irrigated land has a drastically lower agricultural potential, and another 15% is at risk of experiencing the same problem due to soil salinity (Daliakopoulos *et al.*, 2016). The negative impacts of soil salinity on plant health are influenced by various factors, including the sensitivity of the plant, the type and concentration of salt present, agronomic practices, and the presence of additional stress factors. As a glycophyte, rice is extremely sensitive to salty soil (Ma *et al.*, 2018), and it can suffer yield losses of up to 70% when under salt stress conditions, although negative impacts can already be observed during the early stages of seed germination (Farooq *et al.*, 2021).

Plants exposed to high concentrations of salt exhibit stunted growth, reduced leaf size, and pale colouration. The plant's response to salinity involves a decrease in growth and yield due to osmotic adjustment and salt-specific reactions (Kamran *et al.*, 2020). Reduced water absorption ability is associated with osmotic adjustment, while salt-specific reactions are associated with enzyme activity and metabolic changes, hormonal imbalances, membrane transport of water and nutrients, and alterations in ion concentration, leading to toxicity. Salinity stress in plants also causes water scarcity, reduced photosynthesis, oxidative and osmotic stress, and imbalanced nutrient levels (Santos *et al.*, 2022). The K^+/Na^+ ratio is crucial for plants, and salt stress disrupts this balance, leading to reduced nutrient availability (Kumari *et al.*, 2021). Moreover, salt stress can lead to the generation of reactive oxidative species (ROS), resulting in DNA damage and impaired protein metabolism (Hasanuzzaman *et al.*, 2021).

Sustainable agriculture is an essential practice in ensuring food security for the present and future generations. Traditional agricultural practices often rely on the exploitation of natural resources, leading to environmental degradation and depletion of soil nutrients. In contrast, sustainable agriculture focuses on meeting the current needs without compromising the ability of future generations to meet their own needs (Tallapragada and Seshagiri, 2017). One of the primary goals of sustainable agriculture is to boost crop productivity on existing agricultural land instead of expanding the arable landmass. This approach can be achieved by improving soil health, reducing soil pollution, and combatting soil salinization and desertification (FAO, 2018). Biotechnological technologies are currently being explored as a means of improving crop productivity and soil health. One such approach is the use of beneficial soil microorganisms that can interact with plants to promote their growth and protect them from diseases (Suman *et al.*, 2022). These microorganisms can improve soil health by enhancing nutrient availability, promoting soil structure, and reducing soil erosion. Furthermore, they can reduce the need for synthetic fertilizers and pesticides, thereby reducing the environmental pollution. Overall, sustainable agriculture practices are essential for ensuring long-term food security while protecting the environment. The use of biotechnological technologies, such as beneficial soil microorganisms, can enhance crop productivity while promoting soil health and reducing environmental degradation.

Mechanisms Involved in Salt Tolerance of Rice Plants: Implications for Improving Rice Production Under Salinity Stress

As above mentioned, rice plants are classified as glycophytes, which are characterized by their low tolerance to salinity stress and poor physiological and molecular traits. However, rice is often used as a model crop for studying the physiological, biochemical, and molecular responses to salinity stress due to its widespread cultivation and economic importance (Islam *et al.*, 2018). Even being considered a salt-sensitive crop, rice presents different genotypes with different responses when subjected to salt stress. For example, ‘Pokkali’ is a salt-tolerant indica variety, widely used as a donor for breeding programs. On the other hand, there’s another indica variety, IR-29, usually used as a salt-sensitive standard. Additionally, there is also the japonica variety, Nipponbare, that despite not being considered a salt-sensitive standard, is part of the salt-susceptible spectrum (Ferreira *et al.*, 2015).

Understanding the fundamental mechanisms underlying salt tolerance is crucial for enhancing grain yield in rice under salinity stress. Rice plants have two primary mechanisms for coping with salt stress: ion exclusion and osmotic tolerance (Reddy *et al.*, 2017). The process of ion exclusion primarily involves the transport of Na^+ and Cl^- in the roots of rice plants, which helps to prevent the build-up of excess Na^+ and Cl^- in the leaves. This mechanism encompasses the recovery of Na^+ from the xylem, as well as the efflux of ions back into the soil (Liu *et al.*, 2019). As for osmotic tolerance, the plant uses long-distance signals that reduce shoot growth and are activated before the accumulation of Na^+ in the shoot. This is directly connected with the plant's ability to withstand drought-like conditions due to salinity stress. Finally, tissue tolerance happens by sequestration of Na^+ in the vacuole and the synthesis of ROS detoxication enzymes and compatible solutes (Zhao *et al.*, 2021). Osmolytes, including proline, glycine

betaine (GB), and trehalose sugar, have been studied to maintain the osmotic imbalances between the surrounding cells and the cytosol during salt stress (Per *et al.*, 2018). In order to facilitate protein function and stress signalling, they also support the regulation of protein folding. The plants can adapt to unfavourable soil conditions thanks to these reactions (Singh, Kota and Flowers, 2021).

Salt tolerance is a complex quantitative trait that is regulated by multiple genes (Lang *et al.*, 2017). It can also activate signalling pathways that lead to changes in gene expression, and the production of stress-responsive proteins (Razzaque *et al.*, 2019). Under salt conditions, a series of important genes become active, including those related to membrane transport such as OsSOS1, OsNHX1 (Na^+/H^+ antiporters) (Fukuda *et al.*, 2004), OsHKT2;1 (Na^+/K^+ symporter) (Islam *et al.*, 2016), and OsCAX1 (H^+/Ca^+ antiporter) (Kumar *et al.*, 2013), as well as channel proteins such as OsAKT1 (K^+ inward/rectifying channel) (Yang *et al.*, 2014), OsKCO1 (K^+ outward-rectifying channel) (Kumar *et al.*, 2013), OsTPC1 (Ca^{2+} permeable channel) (Kurusu *et al.*, 2012), and OsCLC1 (Cl^- channel) (Diédhiou and Golldack, 2006). In addition to their roles in transport, some of these genes also combat the aversive effects of hydrogen peroxide, such as OsAPX1 (hydrogen peroxide removal) (Lu, Takano and Liu, 2005) and OsCATA (catalase isozyme A synthesis) (Jung *et al.*, 2021). Other genes are involved in water stress, phytohormone regulation, transport, and compound synthesis, including OsRAB21 (water stress-inducible protein) (Mundy and Chua, 1988), OsBZ8 (ABA-responsive element) (Mukherjee *et al.*, 2006), OsNRT1;2 (nitrate transporter) (Wang *et al.*, 2012), and OsP5CS1 (proline biosynthesis) (Bagdi *et al.*, 2015). Overall, the mechanisms that rice plants use to cope with salt stress are complex and involve a variety of physiological, biochemical, and molecular processes. Understanding these mechanisms can help to develop new strategies for improving rice production under saline conditions.

Bacterial Adaptations to Salinity Stress and Their Potential Role in Improving Plant Tolerance

Besides plants, bacteria have also developed diverse mechanisms to adapt to salinity stress, some of which are relevant to enhancing plant tolerance (Etesami and Glick, 2020). Depending on their response to salt, bacteria are categorized as halotolerant or halophilic. Halotolerant microorganisms can survive in high concentrations of NaCl, with or without the presence of salt in the environment and can tolerate it. On the other hand, halophiles are found in salt-rich ecosystems and are naturally occurring communities that require Na^+ for their metabolism and growth, making salt a necessity for their survival (Etesami and Glick, 2020). Numerous bacterial species have been found to tolerate high NaCl concentrations, including *Arthrobacter sp., Azospirillum brasilense, Azospirillum lipoferum, Azotobacter chrooccocum, Bacillus amyloliquefaciens, Bacillus firmus, Bacillus safensis, Bacillus subtilis, Curtobacterium flaccumfaciens, Kocuria rhizophila, Ochrobactrum intermedium, Pseudomonas fluorescence, Pseudomonas putida,* or *Pseudomonas stutzeri* (Shilev, 2020).

To survive in the presence of salinity, halotolerant bacteria have developed a variety of mechanisms, including their cell wall biogenesis and ion-pumping capacity (Ilangumaran and Smith, 2017). These mechanisms involve the production of extracellular polymeric substances (EPSs) to support biofilm formation and limit salt entry into the cell (Morcillo and Manzanera, 2021). Additionally, microorganisms generate soluble salts through protein and enzyme adaptation, as well as optimize intracellular Na^+ ion concentration using the Na^+/H^+ antiporter located in the cell membrane. This antiporter pump's crucial function is to maintain a high K^+/Na^+ ratio within the cytosol compared to the low value in the surrounding environment (Etesami and Glick, 2020). Moreover, the unique structure of the cell wall, including structural and integral proteins, lipid composition, and polysaccharides, helps prevent high salt concentrations from entering the cell (Shahzad *et al.*, 2017). Finally, bacteria can synthesize amino acids and solutes internally as an adaptive mechanism (Ilangumaran and Smith, 2017).

Enhancing Plant Salt Tolerance: The Mechanisms and Benefits of PGPB

Soil salinity not only hinders plant growth but also disrupts the regular plant-microbe interaction in soil, which has a significant impact on plant tolerance to salt stress (Kumar *et al.*, 2021). Plant-microbe interactions are essential in upholding microbial diversity, soil characteristics, and crop productivity during stressful conditions (Kumar *et al.*, 2020). This co-evolution of plants and microbes has resulted in the emergence of facultative intracellular endophytes, to which PGPB belong (Hardoim *et al.*, 2015). Hence, PGPB are a set of advantageous microorganisms that inhabit the rhizosphere, or the soil encompassing plant roots, that can beneficially interact with plant hosts. These microorganisms have developed a variety of mechanisms to enhance plant growth and help the plant cope with a diversity of biotic and abiotic stresses, including salinity (Figure 1) (Souza, Ambrosini and Passaglia, 2015). The mechanisms underlying the growth enhancement and development facilitated by PGPB are diverse and can be categorized by direct and indirect mechanisms.

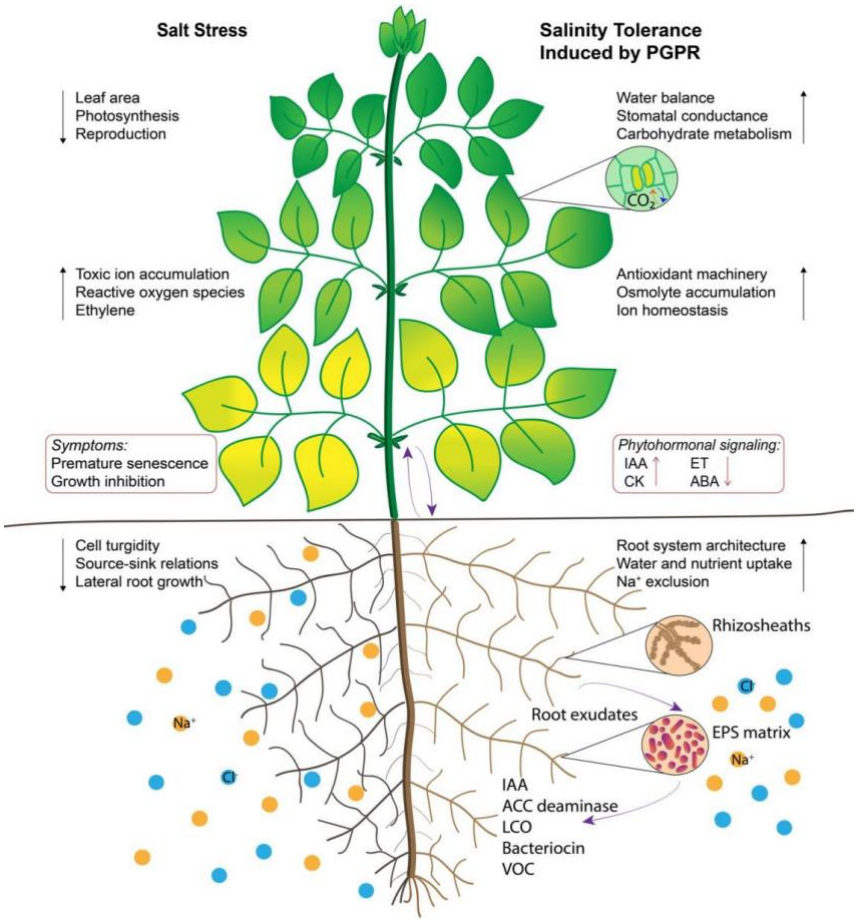

Figure 1. Illustration of salt tolerance mechanisms induced by PGPR

Source: (Ilangumaran and Smith, 2017)

Direct mechanisms refer to the bacterial traits that directly facilitate plant growth by promoting various processes. These include the production of hormones such as auxins, cytokinin, and gibberellin, as well as the utilization of ACC deaminase to regulate ethylene levels. Additionally, bacteria can assist in nutrient acquisition through nitrogen fixation and phosphorous solubilization, as well as iron sequestration by bacterial siderophores (Olanrewaju, Glick and Babalola, 2017).

For example, PGPB can produce phytohormones, which play an essential role in improving plant resilience under salt stress conditions (Kudoyarova *et al.*, 2019). In a study by Ilyas et al., (2020), the authors revealed the inoculation of *Triticum aestivum* by the consortium consisting of *Bacillus sp., Pseudomonas. stutzeri, Azospirillum. brasiliense* and *A. lipoferum* was able to induce salinity tolerance and increase plant biomass and relative water content. The consortium showed a higher production of Indole-3-acetic acid (IAA), gibberellic acid, cytokinin, and abscisic acid under saline conditions, which helped improve plant growth and survival.

Ethylene has both beneficial and harmful effects on a plant's ability to survive in harsh environments. When plants are exposed to salty conditions, the amount of ethylene they produce can be reduced by a substance called 1-aminocyclopropane-1-carboxylate deaminase (ACC-deaminase) which is produced by various beneficial microorganisms. ACC-deaminase breaks down 1-aminocyclopropane-1-carboxylate (ACC) into ammonia and *α*-ketobutyrate, which lowers the amount of ethylene in the plants (Shekhawat *et al.*, 2023). According to a report by Gupta and Pandey, (2019), the PGPB consortium consisting of *Aneurinibacillus aneurinilyticus* and *Paenibacillus sp*. showed a higher ACC deaminase activity. This consortium was able to reduce stress-induced ethylene levels by approximately 60% and helped to lessen the harmful effects of salinity stress on French bean seedlings. These bacteria also led to a significant increase in the length and weight of the roots and shoots, as well as the biomass of the roots and shoots. Additionally, they increased the total chlorophyll content of the seedlings by approximately 57%.

Salinity is a major environmental stressor that negatively affects plant growth and development by disrupting the acquisition of mineral nutrients and interfering with metabolic processes (Shrivastava and Kumar, 2015). However, symbiotic nitrogen fixation by rhizobia that can tolerate salt is crucial for balancing the plant's nitrogen needs under salt stress. Additionally, PGPB can enhance plant resilience to salt stress by converting insoluble phosphorus (P) to an accessible form and increasing the amount of iron available in saline soils by producing siderophores (Sultana, Alam and Manjurul, 2021; Wekesa *et al.*, 2022). Research has shown that bacterial strains such as *Azotobacter chrooccocum, Bacillus sp.* and *Arthrobacter pascens* can improve plant growth and mineral nutrition by fixing nitrogen and solubilizing phosphate under salt stress in maize plants (Ullah and Bano, 2015). Inoculation with these strains partially alleviated saline stress and improved plant growth.

Indirect mechanisms such as biofilm formation and the production of exopolysaccharides (EPS) are also important for aiding plants in saline conditions. Enhanced EPS production due to salt stress leads to biofilm formation, which helps bacteria and their plant hosts by maintaining a water layer around the cells and improving cell adhesion (Morcillo and Manzanera, 2021). Bacteria can also produce substances like antibiotics and cell wall degrading enzymes and compete with pathogenic organisms for resources. Moreover, bacteria can induce systemic resistance in plants and suppress pathogen signaling through quorum quenching (Olanrewaju, Glick and Babalola, 2017). Several bacterial strains have been shown to promote plant growth and stress tolerance under salt stress. For example, *Brevibacterium sediminis, B. linens,* and *Bacillus pumilus* have been reported to enhance plant growth parameters, total proline and glycine betaine accumulation, and antioxidant production in rice plants under salt-stress conditions (Khan *et al.*, 2016; Ahmed *et al.*, 2021; Kumar *et al.*, 2021; Rahman *et al.*, 2022). Additionally, *B. pumilus* has been found to produce strong biofilm, enhance EPS and IAA production, increase ACC-deaminase activity, and solubilize phosphate under salt stress, leading to reduced antioxidant enzyme activity and malonaldehyde content in wheat plants (Ansari, Ahmad and Pichtel, 2019). Overall, bacterial inoculation has emerged as a promising tool for improving crop productivity and stress tolerance in the face of increasing environmental challenges, such as salinity.

The Impact of Environmental Stress on DNA Methylation in Plants: Implications for Adaptation and Gene Expression

Plants are exposed to various environmental changes, such as high temperatures, drought, salt, and heavy metals, which can alter gene expression through epigenetic mechanisms. Epigenetics is the study of changes in gene expression that are not caused by changes in the DNA sequence itself, but by various environmental factors (Miryeganeh, 2021). Recent studies have revealed that many epigenetic modifications occur in plants when they are exposed to stressful environments. These modifications can include changes in chromatin structure, DNA methylation, histone modification, and small RNA expression patterns (Brukhin and Albertini, 2021).

DNA methylation is one of the most important and well-studied epigenetic modifications. It involves the addition of a methyl group to cytosine residues in DNA, which can influence gene expression by blocking the binding of transcription factors and other regulatory proteins to the DNA (Kumar and Mohapatra, 2021). Furthermore, DNA methylation can also be inherited across generations and play a role in plant adaptation to salinity (Sun, Zhuo and Duan, 2022). Several studies have shown that DNA methylation plays an essential role in plant responses to abiotic stresses, including salinity (Akhter *et al.*, 2021; Sun, Zhuo and Duan, 2022; Yung *et al.*, 2022). Genome-wide DNA methylation can be precisely profiled by next-generation sequencing (Barros-Silva *et al.*, 2018). On the other hand, at individual loci, the most common methods for obtaining data on DNA methylation are bisulfite sequencing and methyl-sensitive restriction enzymes (MSRE) analysis, such as Chop-PCR (Li and Tollefsbol, 2011; Šestáková, Šálek and Remešová, 2019).

Chop-PCR is a useful technique for studying DNA methylation in plants because it is relatively simple and cost-effective compared to other methods like bisulfite sequencing. Additionally, it can be used to analyze specific regions of the genome, which can be helpful in the identification of epigenetic changes in response to environmental stresses (Zhang *et al.*, 2014). The Chop-PCR technique can provide information on the methylation status of a particular genomic region in different samples, and its outcome can be analyzed by comparing the intensity of the amplified product between digested and undigested samples (Dasgupta and Chaudhuri, 2019). Overall, Chop-PCR is a valuable tool for studying DNA methylation in plants and can provide insights into the epigenetic mechanisms underlying plant responses to environmental stresses.

Objectives

In this project, our main goal was to isolate seed-borne and endosphere bacteria from salt-tolerant rice plants to use them as biotreatments to increase salt-tolerance capacity in salt-sensitive rice plants.

To cope with this, we divided the main objective into three sub-objectives:

- Have a better understanding of the seed-borne and endophytic microbiota population profiles of three rice cultivars with different responses and sensitivities to salt stress to reveal possible factors contributing to the differing microbiota-mediated responses to salt stress.

- Isolate and screen the best candidates from tolerant cultivars for use as biotreatments against salt stress

- Evaluate the performance of selected bacteria as biotreatments in a hydroponic system.

Finally, because of the lack of knowledge in this field, we decided to go a step further and take the first approach to reveal the putative role of selected strains at the DNA methylation level of the plant when subjected to salinity conditions (epigenetic regulation during the interaction and tolerance regulation process).

Materials and Methods

Plant material

Rice (*Oryza sativa* L.) varieties Pokkali (POK), IR-29 and Nipponbare (NIP) were selected for this study. They were kindly provided by the research group GPlantS at the Institute of Chemical and Biological Technology António Xavier, Portugal (ITQB). The hulls were removed from the seeds by mechanical friction and storage under dry conditions for better preservation.

Endophyte isolation and cultivation

Culturable endophytes from the seed

The germination of twenty-four seeds from each line was performed following a modified method described by Ferreira et al., (2015). Shortly the seeds were surface sterilized by immersion in 70% ethanol for 10 min. with continuous shaking, followed by four washes with sterile double-distilled water (dd H_2O). Thereafter, they were incubated at 28 ± 2°C in the dark on phytoagar (Duchefa Biochemie) squared plates. The culturable seed microbiota was assessed when the roots acquired a length of 5-10 cm. Moreover, the roots were cut, crushed and diluted in a sterile 0.45% NaCl saline solution. In total, four 1/10 dilutions were performed and plated drop-by-drop on LB Broth (Miller's modification) (Thermo Scientific) solid plates, as shown in Fig. 2A. Individual bacterial colonies were selected for further purification by morphological characterization on LB Broth (Miller's modification) solid media (Figure 2B). The selection was based on different morphologies, with colour, size, or brightness as the main criteria. The total population and strain population were counted as colony-forming units (CFUs) per milligram of dry weight (DW) material in order to analyze the relevance, proportions, and prevalence of each.

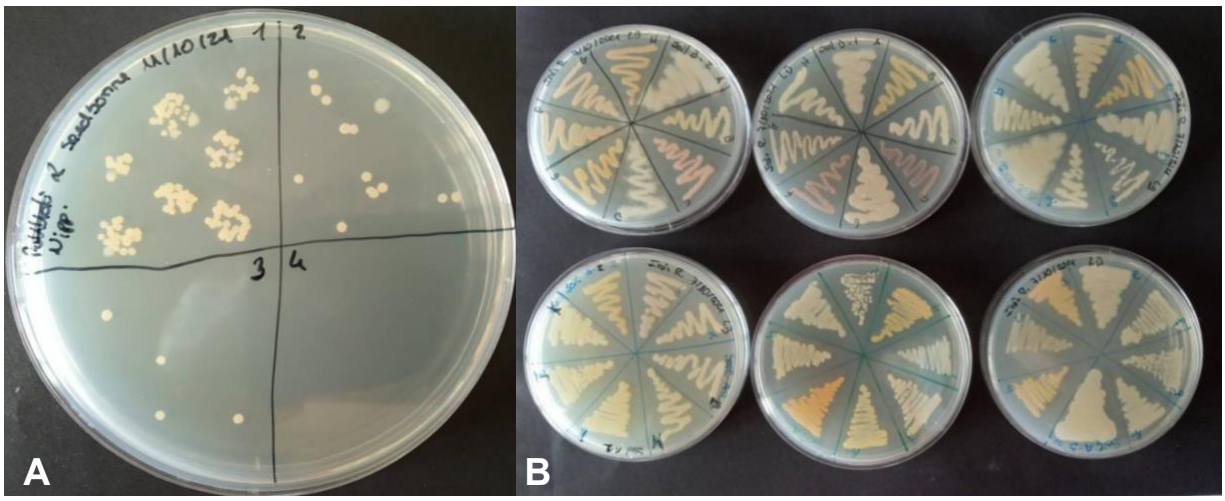

Figure 2. Culturable microbiota accession method

A - Dilution plate schematic; B - Individualized pure cultures.

Culturable endophytes from the plant roots

In order to mimic, as possible, the rice growth conditions, this assay was performed under greenhouse conditions. The soil used in this study was sampled from paddy fields of Centro Operativo e Tecnológico do Arroz (COTArroz), Salvaterra de Magos. Rhizospheric soil was collected on October 4th, 2021, from the upper 15 cm of the soil at three separate locations within the field. Soil culturable microbiota was assessed in accordance with the protocol described in the section “Culturable endophytes from the seed,” taking into consideration that the samples were crushed with a sterile 0.45% NaCl saline solution. These populations were characterized and considered the core population from which roots may be recruited.

The rhizospheric soil was placed in 2 L polythene pots and covered with vermiculite as an aeration layer. The pots were placed in trays to maintain water in the flooding system, as culture conditions are required. One-week-old seeds were germinated as described in the section “Culturable endophytes from the seed”. Seedlings were then individually placed in each pot. Each Oryza sativa accession was represented by 15 pots arranged in a randomized block design (Figure 3). Plants were grown in a greenhouse for four weeks or until two real leaves were obtained.

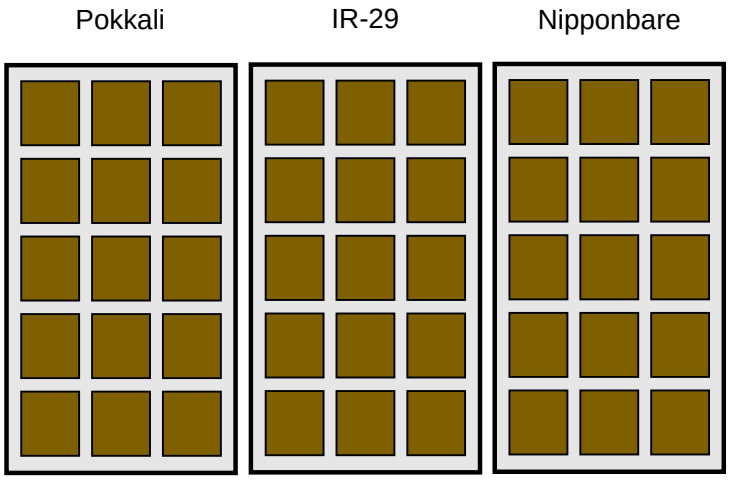

Figure 3. Schematic of randomized block design.

Schematic representation of the boxes containing each rice cultivar (Pokkali, IR-29, Nipponbare). Each box contained 15 pots arranged in a randomized order.

After four weeks, the plants were unearthed, and the roots were washed carefully. Thereafter, the samples were cut into 1 cm pieces, surface-sterilized by immersion in 70% ethanol for 1 min and washed three times with sterile dd H_2O. The plant root microbiota of each cultivar was accessed in accordance with the protocol described in the section “Culturable endophytes from the seed”.

Identification and taxonomical analysis

Isolated strains were identified by polymerase chain reaction (PCR) amplification. For identification, bacterial DNA was extracted using a fast heat shock treatment. Briefly, a single colony of each strain was homogenized in sterile dd H_2O and incubated for 10 min. at 95°C. After incubation, the disrupted cells were centrifuged for 5 min at 12,300 rpm in an Eppendorf 5415D centrifuge. Thereafter, the supernatant was collected and stored at -20°C. After quality verification using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer, DNA was used as a template to perform PCR amplification of the V5-V8 hypervariable region of the bacterial 16S ribosomal RNA gene, for which we used the universal primers 799F (5′- AACMGGATTAGATACCCKG -3′) and 1392R (5′- GGTTACCTTGTTACGACTT-3′). For running, a thermocycler (T100™ Thermal Cycler, Bio-Rad) started with an initial denaturation phase at 95°C for 2 min, followed by 30 amplification cycles at 95°C for 30 s, annealing at 50°C for 30 s, and finally a phase extension at 72°C for 1 min. The final polymerization extension step was performed at 72°C for 7 min. PCR amplifications were all verified by electrophoresis running of the samples in 1% (w/v) agarose gel electrophoresis in 1X TAE (40 mM Tris-HCl, 20 mM, 1 mM EDTA) and 0.05 µL/mL RedSafe DNA Stain was added to the agarose solution. For DNA visualization, 1x Purple loading dye (NEB) was added to each sample before gel loading. GeneRuler DNA Ladder Mix (Thermo Scientific) was used as a molecular weight marker. Electrophoresis was performed at 135 V for 20 minutes. Visualization under UV light revealed bands, and those showing amplification were sent to GENEWIZ (Azenta Life Sciences). The sequences of sufficient quality were compared in the Basic Local Alignment Search Tool (BLAST) database to be identified by 97-100% identity.

Phylogenetic analysis

To study the evolutionary history and taxonomic relationships among bacterial species isolated from diverse origins, we compared the 16S rRNA gene sequences of newly isolated strains with those in the GenBank database using ClustalX. We used ClustalX 2.1 for alignment and tree construction. To visualize the trees, we used the online tool iTOL v6 from the EMBL website, which allows for aesthetic customization of the trees. We generated two different phylogenetic trees based on two different sources of data: endophyte microbiota from seeds (seedborne) and root endophyte microbiota recruited from rice paddy soil (root endophyte).

Evaluation of PGP properties of selected bacterial isolates under salt conditions

PGPB screening is a crucial process that involves identifying and selecting beneficial bacteria that can promote plant growth, increase stress tolerance, and foster sustainable agriculture practices (Pathania *et al.*, 2020). After the isolation and identification of bacterial isolates, eight strains were chosen based on their relation to the salt-tolerant variety Pokkali or their potential to perform well under salt conditions, as reported in the literature. The selected isolates underwent evaluation to determine their growth capacity under varying NaCl concentrations, biofilm formation, auxin production, and ACC deaminase activity.

Bacterial isolates growth conditions

The pure bacterial isolates selected for screening were pre-grown overnight in 15 mL tubes containing LB broth (Miller's modification) at 28 ± 2°C and 180 rpm. For all biochemical assays, 96-well microplates were used as the main system. Therefore, the isolate suspensions were adjusted to an optical density (OD) of 0.05 at 600 nm using the pre-culture grown overnight. Dilutions were made based on volumetric/concentration formulas specific to 96-well plates:

$$Ci \times Vi = Cf \times Vf;$$

$$Vi\ (\mu L) = (200\ \mu L \times 0.05\ OD_{600\ nm}) / \text{Pre-culture } OD_{600\ nm}$$

The Thermo Scientific Multiskan SkyHigh Microplate Spectrophotometer was used to measure the absorbance required for each test result. When required, the Eppendorf 5810R centrifuge was used for plate centrifugation. All assays were performed using LB Broth (Miller's modification) containing the regular NaCl concentration for this broth (0.09 mM), except for the ACC deaminase activity assay, for which a minimal medium M9 was used. A NaCl concentration of 0.5 mM or a range of increasing concentrations (0.5 mM, 1 mM, and 2 mM) was used as the imposed salt stress. Five replicates were used for each assay, and a proper control was included in each test.

Growth under salt

In order to determine the growth performance of the isolates under different salt conditions, the 96 well plates were incubated at 28 ± 2°C and 180 rpm for 24 hours. The absorbance of each well was measured at 600 nm at 3, 6, 12, and 24 hours. Growth curves were prepared for each salt condition and compared to determine the effect of salt on the growth of each strain.

Biofilm formation

The biofilm formation test was carried out using a modified method by Saravanan, Radhakrishnan and Balagurunathan, (2015). First, the 96-well plates were incubated at 28 ± 2°C and 180 rpm for 48 hours. After the incubation period, the medium was removed, and the plate was washed to remove all planktonic structures. The remaining material was considered as biofilm structures and was stained with a 0.5% crystal violet solution (prepared with 500 g methyl violet, 25 mL methanol, and 75 mL H_2O) for 10 minutes. Then, the excess staining solution was washed away, and 30% glacial acetic acid was added to dissolve all the stained structures. After incubating for 10 minutes at room temperature, the absorbance was measured at 550 nm.

Auxins production

Auxin production was assessed using a modified method by Sarwar et al., (1992). Briefly, LB Broth (Miller's modification) was supplemented with 5 g/L of L-tryptophan. The 96 well plates were incubated at 28 ± 2°C for 48 h and 180 rpm. After incubation, the plates were centrifuged at 3800 rpm for 50 min and 100 µL of the supernatant was transferred to a fresh plate. Then, Salkowski's reagent (For 50 mL; 1 mL of 0.5 M $FeCl_3$ and 49 mL of 35 % $HClO_4$), and the plates were left at room temperature for 20 min, until the emergence of pink color. The absorbance was measured at 530 nm, and the quantification of auxin equivalents (in µg/mL) was performed using the calibration curve presented in Annex 1.

ACC deaminase activity

The ACC deaminase production was indirectly assessed using a modified method by Naveed et al., (2008) Briefly, 96 well plates with minimal medium M9 (composition in Annex 2) supplemented with 3 mM ACC were incubated at 28 ± 2°C for 72 h at 160 rpm. After incubation, absorbance was measured at 600 nm.

Inoculation of candidate strains in a hydroponic growth system

Testing bacterial inoculants is crucial for identifying the most effective strains and for developing better formulations that can be used in different agricultural settings by evaluating their efficacy in stimulating plant growth and resistance under various conditions, such as salt stress (Khan *et al.*, 2022). For this assay, POK was used as a control for salt tolerance and NIP was used as the salt-sensitive variety.

Plant material and growing conditions

Seed germination was assessed using a modified method described by Ferreira et al., (2015). Briefly, rice seeds were surface sterilized with ethanol 70% for 10 min, followed by 20% sodium hypochlorite for 3 min, and five washes with dd H_20. Seeds were then placed in a glass Petri dish over moistened paper towels and kept at 28 ± 2°C in the dark for 24-48 h.

The growth conditions were set according to the method described by Almeida et al., (2016). Succinctly, the plants were placed in a plant growth chamber, where the temperature was maintained at 25 ± 2°C, with a 12 h photoperiod, under full spectrum light (95-130 µmol/m^2) and relative humidity of 60-70%. The plants were grown in Yoshida nutrient solution (1X) (composition in Annex 3) under control conditions, and in saline Yoshida nutrient solution (1X) obtained by the addition of 3M NaCl until the required electrical conductivity (EC) was achieved. Throughout the experiment, the nutrient solution was replaced every eight days and adjusted daily to a constant pH of 5.1.

Bacterial inoculum and plant inoculation

Bacterial inoculation was performed following the methodology described by (Vílchez *et al.*, 2021). In summary, the selected strains were individually grown in a 1 L Erlenmeyer flask at 28 ± 2°C for 10-14 h and 180 rpm to obtain a bacterial density of 10^4 CFUs/mL. The inoculum was centrifuged at 10000 rpm for 7 min using an Avanti J-26 XPI centrifuge (Beckman Coulter). The pellet was resuspended in Yoshida nutrient solution (1X) to maintain the bacterial density. Salt-sensitive plants (NIP) were treated with the candidate strains by submersion in the inoculum for 10-12 h.

Experimental design

The hydroponic system was assembled following the method described by Almeida et al., (2016), with specific modifications. In summary, the system included two main components. The first one was the plastic pots with a volume of 700 mL (Figure 4A). The pots were filled to 650 mL in order to keep the seedlings submerged by only 2 mm. The second component was the seedling Styrofoam floater (Figure 4B). Each floater contained 15 wells, and each well was filled with two seedlings.

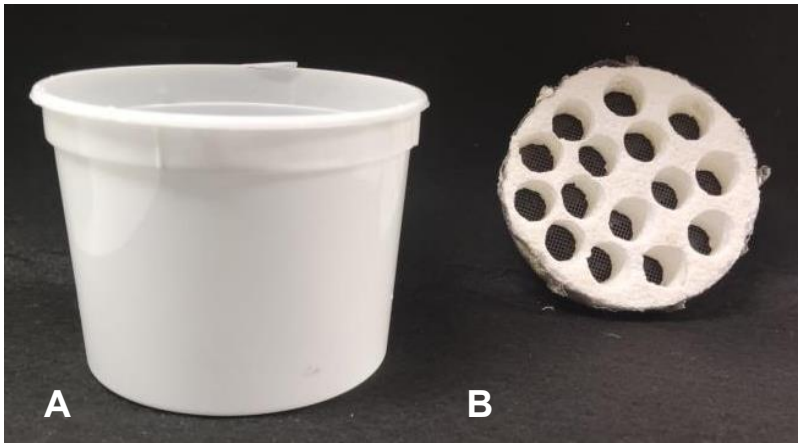

Figure 4. Hydroponic system composition

A. 700 mL plastic pot; B. Seedling Styrofoam floater.

The experiment was conducted in a completely randomized block design with a total of twenty-four pots. Fifteen biological replicas were used per condition: POK, NIP, NIP + Bacteria 1, NIP + Bacteria 2, NIP + Bacteria 3, and NIP + Bacteria 4. This system was applied to each treatment, the control, and the salt. A schematic of the experimental design is presented in Fig. 5. To minimize the effect of eventual differences in chamber conditions, the pot's position was rotated daily.

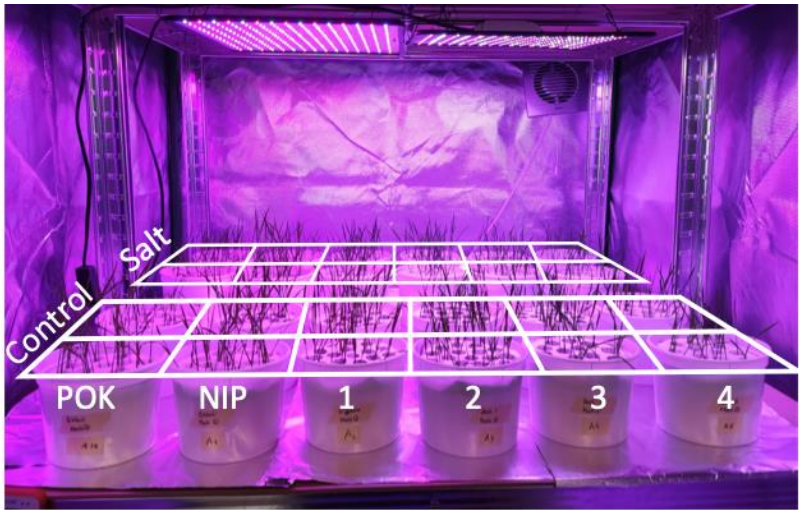

Figure 5. Experimental design schematic

The numbers 1, 2, 3 and 4 represent the treatment with each candidate strain.

The experimental design is illustrated in Fig. 6. In summary, two days after sowing, the seedlings were transferred to the seedling Styrofoam floaters filled with dd H_2O in the dark for three days. Following, from day four onwards, the plants were grown in Yoshida nutrient solution (1X). On day ten, the plants were inoculated as described in the section “Bacterial inoculum and plant inoculation”. After inoculation, Yoshida nutrient solution (1X) was refreshed. On day twelve, salt stress was applied to start with EC = 6 dS/m, and after three days, EC was increased to EC = 12 dS/m. By day twenty-two, one pot from each treatment and condition was taken and sampled for molecular analysis. Finally, on day twenty-eight, the remaining plants were harvested, and a photographic record was taken. For phenotypic analysis, different parameters were observed, such as, shoot height and root length, which were assessed using ImageJ software (Schneider, Rasband and Eliceiri, 2012) (https://imagej.nih.gov/ij/download.html), and total/root DW, which were assessed by leaving the plants in the oven at 70 ± 2°C for 48h.

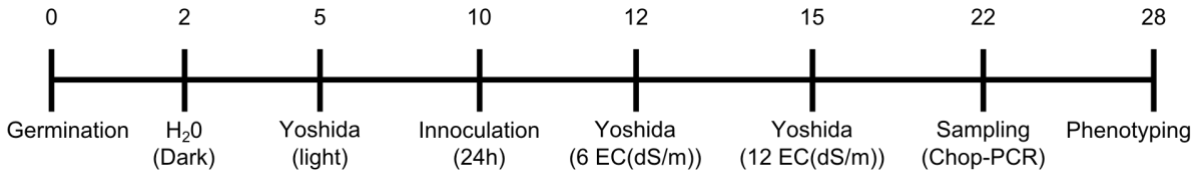

Figure 6. Experimental timeline

The numbers are referent to the number of days after germination (day 0).

Chop-PCR Analysis

DNA purification

Genomic DNA from the roots and shoots under each condition was isolated using the method described by Edwards, Johnstone and Thompson, (1991), with specific modifications. Briefly, the plant material was ground in liquid nitrogen and 50 mg was used for further experiments. To the ground material, 400 µL of DNA extraction buffer (Edwards Solution) was added and the mixture was vortexed. After vortexing, 150 µL of 3 M sodium acetate (pH 5.2) was added and the mixture was vortexed for 10 s. The tubes were then incubated at 4°C for 10 min. After incubation, the tubes were centrifuged at 10,000 rpm for 5 min. The supernatant was collected, and 400 µL isopropanol was added, mixed, and incubated at 4°C for 10 min. The mix was then centrifuged at 10,000 rpm for 5 min. and the supernatant was discarded. Thereafter, washing with 250 µL of Ethanol 70% was performed. To detach the pellet, the tubes were vortexed and centrifuged for 3 min. at 8,000 rpm and the supernatant was discarded. The sediment DNA was then dried at 65°C for 5 min. Finally, DNA was eluted with 50 µL of deionized water and stored at 4°C. DNA quantification was performed using a NanoDrop One/OneC Microvolume UV-Vis Spectrophotometer.

DNA digestion with MSRE

The methylation-sensitive restriction enzyme (MSRE) digestion technique was employed following the procedure described by Zhang et al., (2014). Briefly, DNA [1000 ng/µL] was used as a template for digestions for 1 h with a final volume of 50 µL. Digestion was performed using a single-cut MSRE (*Bsa*AI, *Bst*BI, *Hae*II, *Hha*I, and *Xho*I). The specifications of each enzyme can be consulted in Annex 4. The digestion product was verified by 2% (w/v) agarose gel electrophoresis at 80 V in a sub-cell GT horizontal electrophoresis cell (Bio-Rad). Gel visualization was performed under UV light and images were acquired using Gel Doc XR+ (Bio-Rad).

Primer design and PCR conditions

For all the selected genes (Annex 5), the PCR primers were chosen to flank each enzyme restriction site in the selected regions. Primer design was performed using Primer3 v.0.4.0 software. All primer pairs (Annex 5) were evaluated to identify the optimal amplification efficiency and check for the formation of non-specific products. For amplification, a DNA template (50 ng) and VWR Taq Plus DNA Polymerase Master Mix were used.

Thermocycler conditions started with an initial denaturation at 95°C for 5 min, followed by 34 cycles at 95°C for 30 s, annealing at a range of 57.5 to 60.5°C, depending on each primer, for 30 s, and extension at 72°C for 1 min. The final polymerization extension step was performed at 72°C for 5 min. PCR amplification was verified by 1% (w/v) agarose gel electrophoresis at 120V in a Sub-Cell GT Horizontal Electrophoresis Cell by Bio-Rad and using a 100 bp DNA Ladder (Thermo Fisher Scientific). Gel visualization was performed under UV light and pictures were acquired using the Gel Doc XR+ (Biorad).

Statistical analysis

The collected data were analyzed by two-way analysis of variance (ANOVA) to determine statistically significant differences between the means of treatments according to Tukey's or Šidák's post-tests ($p > 0.05$). All statistical analyses were performed using GraphPad Prism, version 9.0.0.

Results and Discussion

Chapter 1. Diversity and Community Composition of Culturable Seedborne and Root-recruited Microbiota

During the first phase of this project, the culturable seed-borne and root-recruited populations of three rice cultivars (Nipponbare, IR-29, and Pokkali) were evaluated by counting the number of CFUs per milligram of DW of sampled biomass. We must indicate that the only purpose of the second salt-sensitive cultivar, IR-29, is to reveal the possible differences in the microbiota of another sensitive cultivar, not to use it anymore in further studies. The culturable microbiota of each rice cultivar was analyzed using different samples. Thus, the first part targets the plant seeds, and the second part, the culturable microbiota recruited by the rice seedlings growing in rice-field paddy soil.

Culturable endophytes from the seed

The diversity and variability of the seed microbiota can range from one to hundreds of species. Thus, its composition varies between flexible and core microbiota (Abdelfattah *et al.*, 2022). To better understand the endophytic microbiota diversity present in each variety, the seed microbiota was analyzed in several mixes of radicles and secondary roots, trying to isolate the microbiota of the plant.

From the collection of isolated strains, and based on their morphological characteristics, a total of 11 isolates were selected for further study. The phylogenetic tree constructed based on 16S gene amplicon sequences for the 11 isolates is shown in Fig. 7. The highest number of isolates was isolated from the cultivar IR-29 (8 in total), as well as the highest diversity, with six different genera (*Bacillus, Curtobacterium, Enterobacter, Paenibacillus, Pseudomonas,* and *Stenotrophomonas*). On the other hand, the cultivar Nipponbare was the next highest number of isolates (5), while Pokkali obtained only three. Both cultivars consisted of the same two genera (*Bacillus* and *Paenibacillus)*, regarding diversity. Overall, these results demonstrate the presence of abundant and diverse endophytic bacteria within the seeds of all rice cultivars.

Of the 11 isolates, one of them remains unidentified after several tries, due to the lack of enough base pairs for accurate identification. We speculate that this isolate could correspond to yeast or archaea. In total, we identified 10 unique strains (*Bacillus altitudinis, Bacillus cereus, Bacillus pumilus, Curtobacterium flaccumfaciens, Enterobacter asburiae, Paenibacillus pabuli, Paenibacillus polymyxa, Paenibacillus tundrae, Pseudomonas moraviensis,* and *Stenotrophomonas maltophilia*). Among them, as we can see in Fig. 8, five strains were only present in IR-29 seeds (*Bacillus cereus, Curtobacterium flaccumfaciens, Enterobacter asburiae, Pseudomonas moraviensis,* and *Stenotrophomonas maltophilia*), one was exclusively present in Nipponbare seeds (*Paenibacillus tundrae*) and another one only in Pokkali seeds (*Paenibacillus pabuli*), sharing two strains among all (*Bacillus pumilus* and *Paenibacillus polymyxa*). These last two may consist of a core microbiota for these three cultivars. Finally, there was one shared strain between the sensitive cultivars Nipponbare and IR-29 (*Bacillus altitudinis*).

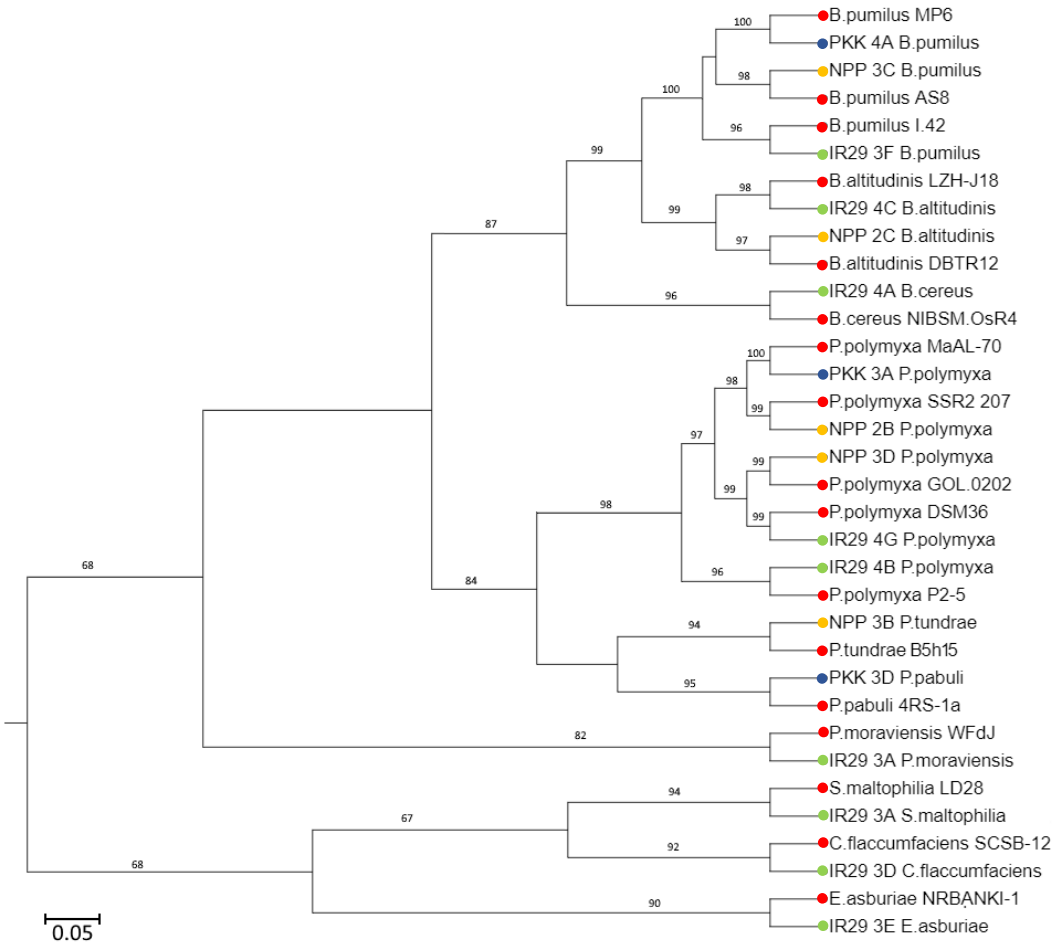

Figure 7. Phylogenetic tree of strains isolated from the seeds of the three different *Oryza sativa* L. cultivars.

The phylogenetic tree shows the related species used to construct the seed-borne collection. In the tree, the orange labels stand for cv. Nipponbare isolates; green, for cv. IR-29 isolates; and blue, for Pokkali isolates. Red labels stand for the control strains determined by BLAST for identified strains. The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site. Numbers on the branches indicate the bootstrap percentage after 1000 replications in constructing the tree.

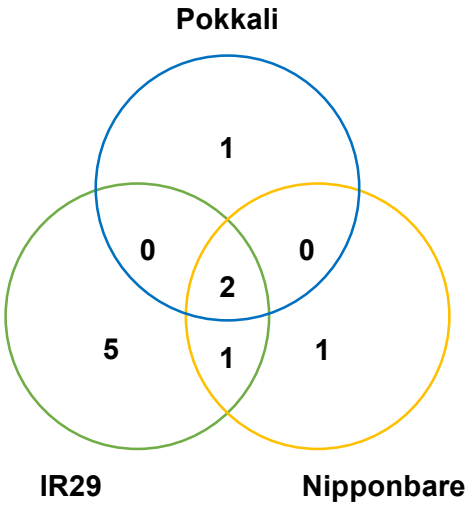

Figure 8. Venn Diagram of strains isolated from the seeds of three different *Oryza sativa* L. cultivars.

The Venn diagram shows the isolated strains that overlap between each cultivar.

According to the counting of the full population present in the seeds of each cultivar, the one with the higher recorded value was the salt-tolerant cultivar Pokkali ($3.44x10^4$ CFUs/mg of DW biomass). On the other hand, the salt-sensitive cultivars presented lower values, where IR-29 showed a 42% less amount on average (2.01), meanwhile, the lowest value was registered in Nipponbare (1.21) with a population about 65% below the one in Pokkali.

To obtain the representative distribution of the microbiota present in the different rice cultivars, a relative population analysis was performed in several tests with different seed batches (Figure 9). Our results show that the most prevalent strain was the same in two of the cultivars, Nipponbare and Pokkali, although with a difference in the relative presence (*B. pumilus* with 39% and 81%, respectively). On the other hand, *B. cereus* represented the most prevalent strain in the IR-29 cultivar (35%). As second most prevalent, all the cultivars presented a different strain and even family, being *B. altitudinis* (20%) in Nipponbare, *C. flaccumfaciens* (24%) in IR-29, and *P. polymyxa* (10%) in Pokkali. Taking into consideration both sensitive cultivars, these shared three strains (*B. altitudinis, B. pumilus,* and *P. polymyxa*), although with differences in prevalence in Nipponbare (20%, 39%, and 3%, respectively) and IR-29 (3%, 2% and 15%, respectively). Finally, the only unidentified strain was present in 38% of the seed microbiota population of the cultivar Nipponbare, hindering the comparisons.

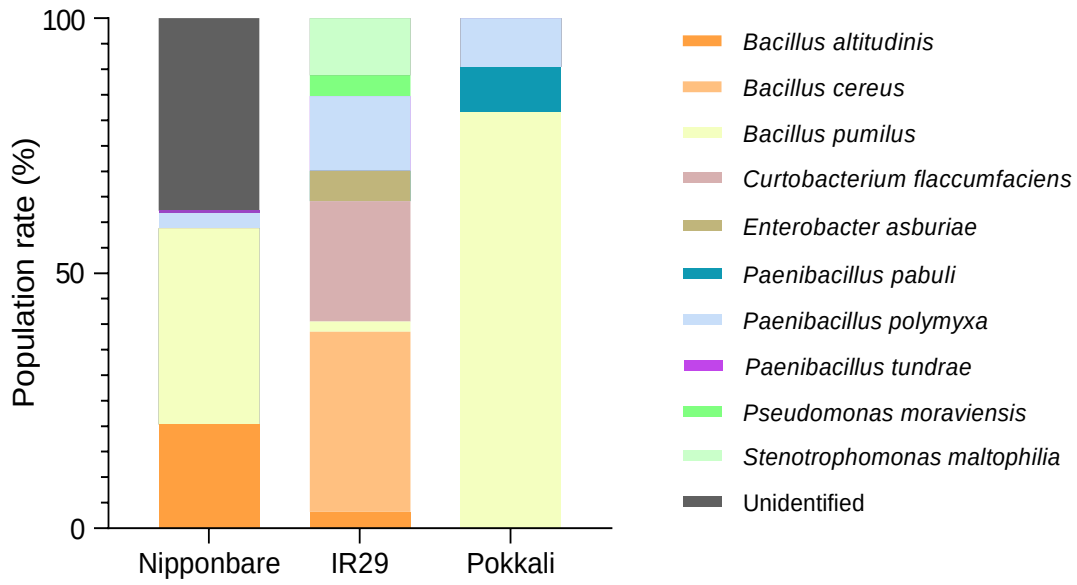

Figure 9. The relative presence of culturable microbiota isolated from the seeds of three different *Oryza sativa* L. cultivars

The graph bar represents the population resilience (%) in the seeds of the rice cultivars Nipponbare, IR-29, and Pokkali under *In Vitro* conditions (n=9). Each strain was identified with a colour as indicated in the legend for all the cultivars.

The results presented here demonstrate that rice seeds harbor a diverse and abundant endophytic microbiota, which varies among cultivars. Previous studies have also shown the presence of endophytic bacteria in rice seeds (Shi et *al.*, 2019; Trivedi et *al.*, 2020), but this study provides additional information on the composition and variability of the seed microbiota. Our findings support the hypothesis that endophytic bacteria are transmitted vertically through seeds and that the seed microbiota contributes to the establishment and maintenance of plant health and growth (Johnston-Monje and Raizada, 2011; Trivedi et *al.*, 2020).

The higher population of endophytic bacteria in the salt-tolerant cultivar Pokkali compared to the salt-sensitive cultivars (IR-29 and Nipponbare) is consistent with previous studies that have reported a positive correlation between salt tolerance and bacterial abundance in plants (Pandey et *al.*, 2016; Singh et *al.*, 2019). These findings suggest that the seed microbiota may play a role in conferring salt tolerance to plants, although further research is needed to elucidate the underlying mechanisms.

The differences in the prevalence of certain bacterial strains among cultivars may also have implications for plant health and growth. For example, *B. cereus* was found to be the most prevalent strain in the IR-29 cultivar, while *B. altitudinis* was the most prevalent in Nipponbare. These strains belong to different bacterial families and may have different functional roles in the plant. Similarly, the unidentified strain present in Nipponbare may represent a novel bacterial species with unique properties that could be of interest to future research.

The identification of 11 unique bacterial strains in the rice seed endophytic microbiota, including 10 identified species and one unidentified strain, sheds light on the diversity and complexity of this community. The identified strains belonged to six different genera (*Bacillus, Curtobacterium, Enterobacter, Paenibacillus, Pseudomonas,* and *Stenotrophomonas*), and were found in varying abundances across the three cultivars analyzed. Among these, *Bacillus and Paenibacillus* were the most common genera, with *B. cereus, B. pumilus,* and *P. polymyxa* being the most prevalent species.

The presence of these bacterial strains in the seed microbiota is likely to play an important role in plant growth and development. For example, *Bacillus* and *Paenibacillus* species are known to produce plant growth-promoting hormones, such as auxins and cytokinins, and to solubilize phosphate, which can improve plant nutrient uptake (Lugtenberg and Kamilova, 2009; Ali et *al.*, 2014). Additionally, many of the identified strains, including *B. altitudinis, B. cereus, P. polymyxa,* and *S. maltophilia*, have been shown to have biocontrol activity against plant pathogens (Santoyo et *al.*, 2012; Wang et *al.*, 2018). Therefore, the presence of these strains in the seed endophytic microbiota may confer some level of protection against pathogen invasion and improve plant health.

It is also worth noting that the abundance and diversity of the seed endophytic microbiota were found to vary between cultivars. Pokkali, a salt-tolerant cultivar, had the highest bacterial population density, while the two salt-sensitive cultivars, Nipponbare and IR-29, had lower densities. This suggests that the seed microbiota may play a role in conferring salt tolerance in plants, as has been previously suggested (Marasco et *al.*, 2012).

Overall, these findings highlight the importance of understanding the diversity and variability of the seed microbiota in different rice cultivars. Further research is needed to determine the functional roles of specific bacterial strains and their interactions with the plant host. This knowledge could be used to develop microbial-based strategies to enhance plant health and productivity in rice cultivation.

Culturable endophytes from the roots

Many different microorganisms communicate and interact with plants based on different mechanisms. The rhizosphere is full of microorganisms as a result of the richness of nutrients compared to bulk soil, which increases these microbial interactions (Yu and Hochholdinger, 2018). These rhizosphere interactions can be modulated by the plant by using different recruiting compounds (Santoyo, 2022). Due to the importance of these specific interactions, the culturable root-recruited microbiota of each rice cultivar was analyzed by growing the rice plants in rice-field paddy soil under greenhouse conditions.

Eighteen seemingly unique isolates were chosen for further investigation from the isolate collection based on their morphology. As in the previous section, a phylogenetic tree was constructed based on 16S gene amplicon sequences of the 18 isolated strains (Figure 10). The microbiota of the rice-field paddy soil sample was used as a control. These samples presented a high number of isolates (14) with a wide range of genera (*Acinetobacter, Aeromonas, Bacillus, Buttiauxella, Exiguobacterium, Pseudomonas, Rossellomorea,* and *Vogesella*). Regarding the three cultivars (Nipponbare, IR29, and

Pokkali), all presented a similar number of isolates (7, 9, and 7, respectively) and genera diversity (*Bacillus, Lysinibacillus, Peribacillus, Priestia,* and *Rossellomorea*). Altogether, we can already see differences between the genera inherited by the seeds and the genera resulting from the early-stage recruiting interactions.

In total, we were able to identify 12 unique strains from the soil sample, including *Acinetobacter guillouiae, Aeromonas veronii, Bacillus altitudinis, Bacillus cereus, Bacillus mycoides, Bacillus Pseudomycoides, Buttiauxella agrestis, Exiguobacterium acetylicum, Pseudomonas fluorescens, Rossellomorea marisflavi, Rossellomorea sp.,* and *Vogesella fluminis*. We also identified another 11 unique strains from all three cultivars, which included *Bacillus altitudinis, Bacillus cereus, Bacillus mycoides, Bacillus pumilus, Bacillus subtilis, Lysinibacillus fusiformis, Lysinibacillus sphaericus, Peribacillus frigoritolerans, Priestia megaterium, Rossellomorea marisflavi,* and *Rossellomorea sp*. In Fig. 11, we can see the overlapping strains between each cultivar, without taking into consideration the soil microbiota in this analysis. Not all cultivars presented unique strains associated with them, as in the case of Pokkali. This did not happen with the sensitive cultivars, Nipponbare (*Peribacillus frigoritolerans, Rossellomorea marisflavi,* and *Rossellomorea sp.)* and IR-29 *(Bacillus pumilus*). Interestingly, among all cultivars, three shared strains were found (*Bacillus cereus, Bacillus mycoides,* and *Priestia megaterium*). Additionally, there were isolates only shared by the sensitive cultivars Nipponbare and IR-29 (*Lysinibacillus sphaericus*) and by IR-29 and Pokkali (*Bacillus altitudinis, Bacillus subtilis, Lysinibacillus fusiformis,* and *Rossellomorea sp*.).

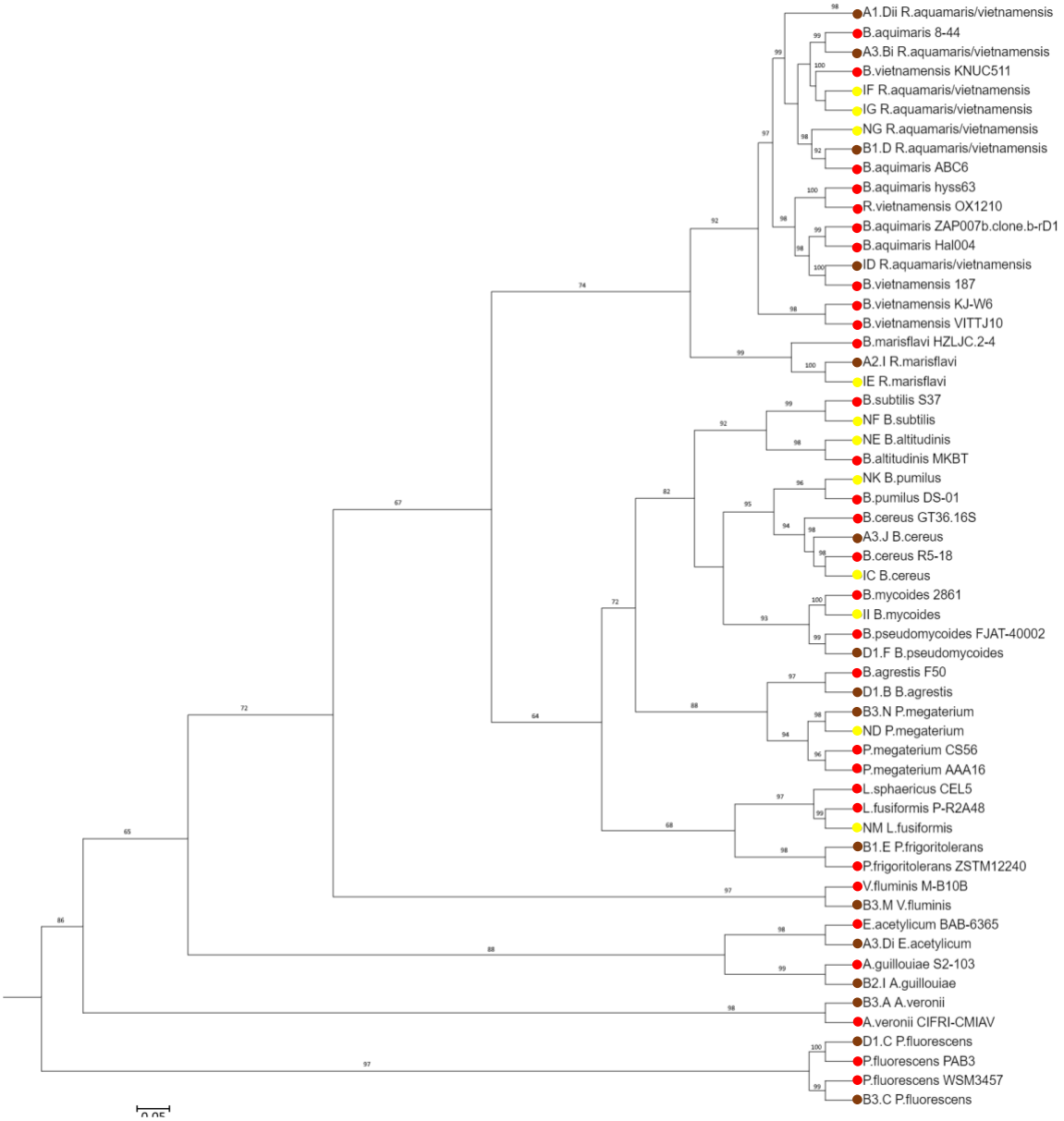

Figure 10. Phylogenetic tree of strains isolated from the roots of the three different *Oryza sativa* L. cultivars

The phylogenetic tree shows the related species used to construct the root-recruited collection. In the tree, the yellow labels stand for the root isolates of the three cultivars; The brown labels for the soil isolates, and the red labels for the control strains determined by BLAST for identified strains. The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site. Numbers on the branches indicate the bootstrap percentage after 1000 replications in constructing the tree.

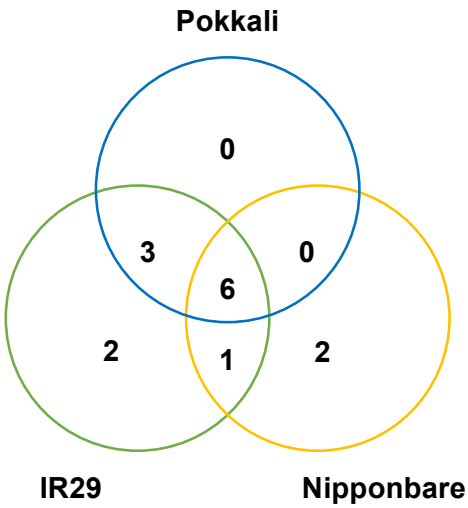

Figure 11. Venn Diagram of strains isolated from the roots of the three different *Oryza sativa* L. cultivars

The Venn diagram shows the strains isolated that overlap in each cultivar.

To obtain a clearer differential pattern in the microbiota recruited by each rice cultivar, we performed this assay with paddy soil from a rice field, providing the plants with the same background microbiota. When we counted the endophytic population from each cultivar, we found that the Nipponbare cultivar had almost double the population ($7.59x10^1$ CFUs/mg of DW biomass) compared to the other two cultivars, IR-29 ($3.96\ x10^1$ CFUs/mg of DW biomass) and Pokkali ($4.58\ x10^1$ CFUs/mg of DW biomass), which showed similar results.

In Fig. 12, we can see a clear pattern between the most prevalent strain, *Rossellomorea sp.*, which occurred in all cultivars and the soil sample (control). Between cultivars, the results showed a common occurrence of two strains (*Bacillus cereus* and *Priestia megaterium*), although they were not present in the control condition with the same prevalence. This might indicate that despite not being relevant enough to be identified in the soil population, the two strains were present in the cultivar, indicating a selective and direct recruiting process. Although with different salt-tolerant capacities, IR-29 and Pokkali presented the same second most prevalent strain (*Lysinibacillus fusiformis*).

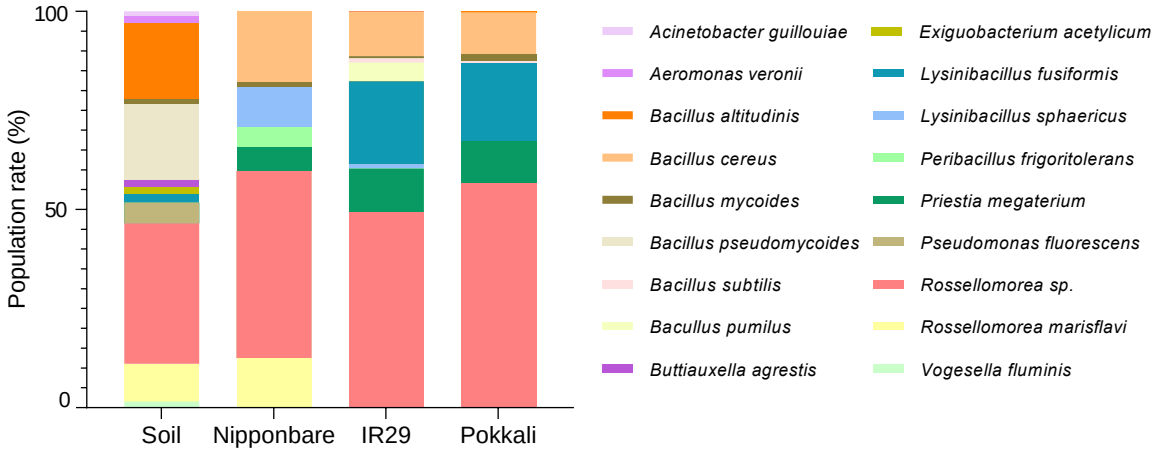

Figure 12. The relative presence of culturable microbiota isolated from the roots of three different *Oryza sativa* L. cultivars grown in rice paddy soil

The graph bar represents the population resilience (%) in the roots of the rice cultivars Nipponbare, IR-29, and Pokkali under greenhouse conditions (n=15) in rice paddy soil. The soil was considered as a control to be used in contrast to the remaining isolated strains. Each strain was identified with a colour as indicated in the legend for all the cultivars.

Result Discussion

The results presented in the text highlight the importance of the rhizosphere and early-stage recruiting interactions in determining the cultivar-specific microbiota of rice plants. The study found that rice plants recruited a unique set of microorganisms from the soil, with different cultivars recruiting different sets of microbial strains. The findings also suggest that plants might play a role in modulating the microbial interactions in the rhizosphere through the use of recruiting compounds. These results are consistent with previous studies that have shown the importance of plant-microbe interactions in determining the plant's health and productivity (Berendsen, Pieterse and Bakker, 2012; Bulgarelli *et al.*, 2012).

Additionally, the findings of this study highlight the importance of considering cultivar-specific microbiota in agricultural practices, as different cultivars might have different microbial communities that affect their growth and yield. The study also identified several bacterial strains that were shared among the different cultivars, indicating their potential importance in rice plant-microbe interactions. For instance, *Bacillus cereus* and *Priestia megaterium* were found in all cultivars, while *Lysinibacillus fusiformis* was shared by two cultivars. Previous studies have also identified these bacterial strains as potential plant growth-promoting microorganisms (PGPMs) (Homthong *et al.*, 2022; Zhang *et al.*, 2022). Thus, the findings of this study provide further evidence for the potential use of these bacterial strains as biofertilizers to improve crop yield.

Overall, the results of this study provide insights into the complex interactions between plants and microorganisms in the rhizosphere and highlight the importance of considering cultivar-specific

microbiota in agricultural practices. Furthermore, the identification of potential PGPMs provides opportunities for the development of novel biofertilizers to enhance crop productivity.

Chapter 2. Evaluation of PGP properties of selected bacterial isolates under salt conditions

Numerous bioactive substances produced by endophytic microorganisms have a variety of biological functions and may either directly or indirectly promote plant growth (Singh *et al.*, 2017). In order to understand the possible role of plant growth promoting (PGP) traits in salt tolerance improvement of salt-sensitive rice plants, a characterization of eight strains selected based on the correlation with the salt-tolerant cultivar Pokkali or previously proven by the literature as PGPB (*B. altitudinis, B. subtilis, E. asburiae, E. acetylicum, L. fusiformis, P. pabuli, P. moraviensis, S. maltophilia*) was performed. The analysis was based on a high-throughput 96-well plate system, using quantitative methods. The strains were grown under non-salt conditions (control) and 0.5 M of salt (treatment).

Growth under salt

To select the best candidates for rice inoculation under saline stress, we must understand their capacity to grow under salt stress. To achieve this, the candidates were grown under three different salt concentrations (0.5 M, 1 M and 2 M) (Figure 13). Our data indicated that, in general, all strains presented positive results under salt stress, which might indicate a potential for all of them as future inoculates. However, there were two outstanding strains. Obtaining the best results at 1 M resulted in NF_*B. subtilis* with a growth of 0.68 OD, followed by NE_*B. altitudinis*, presenting positive growth (0,.25) under 2M salt concentration. Four of the selected strains showed an increase in growth compared to the control condition (I3E_*E. asburiae*, P3D_*P. pabuli*, and I3A_*P. moraviensis*). Finally, the strain I3B_*S. maltophilia* even though not presenting the best results in high salt concentrations, was able to maintain the same growth under the control and 0.5 M.

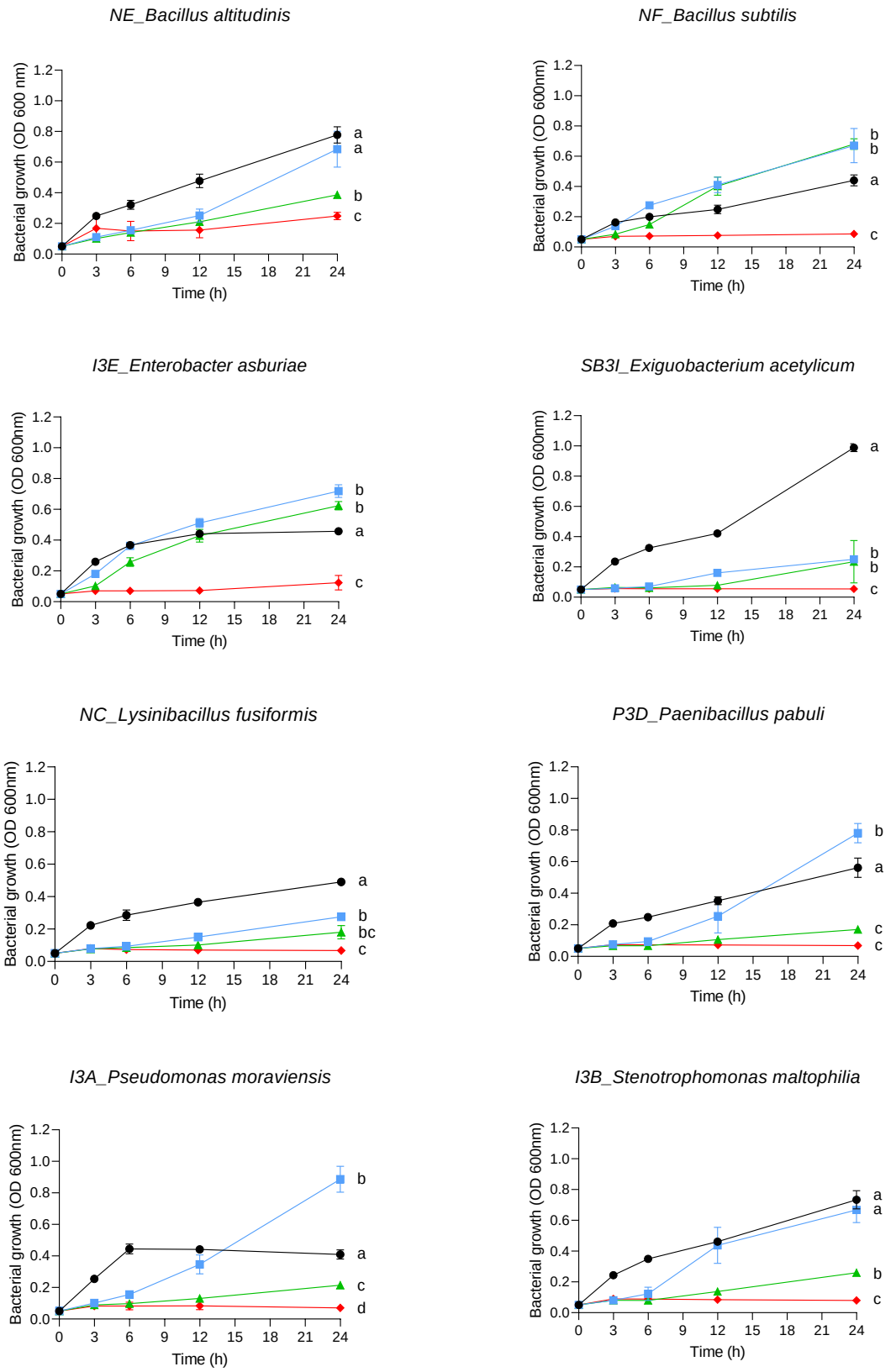

Figure 13. Effect of different NaCl concentrations on bacterial growth

The line graphs represent the bacterial growth of eight strains. In the graphs, the black lines stand for the control conditions (without salt); The blue lines stand for 0.5M of NaCl; The green lines stand for 1M of NaCl, and the red lines stand for 2M of NaCl. Each value is the mean of three replicates. Error bars represent ± standard deviation.

The sets of data were compared by two-way ANOVA test and 95% confidence intervals, where different letters represent significant statistical differences based on Tukey post-tests.

Biofilm formation

Bacteria that live in biofilm are more protected from harmful environments (Yin *et al.*, 2019). Moreover, is one of the main mechanisms that they can use for protecting roots from salt stress. In order to estimate the survival capacity and the potential production under stressing conditions of each candidate strain, is necessary to understand their capacity for biofilm formation in saline conditions. The results comparing the biofilm formation of each candidate with its respective control are shown in the Fig. 14.

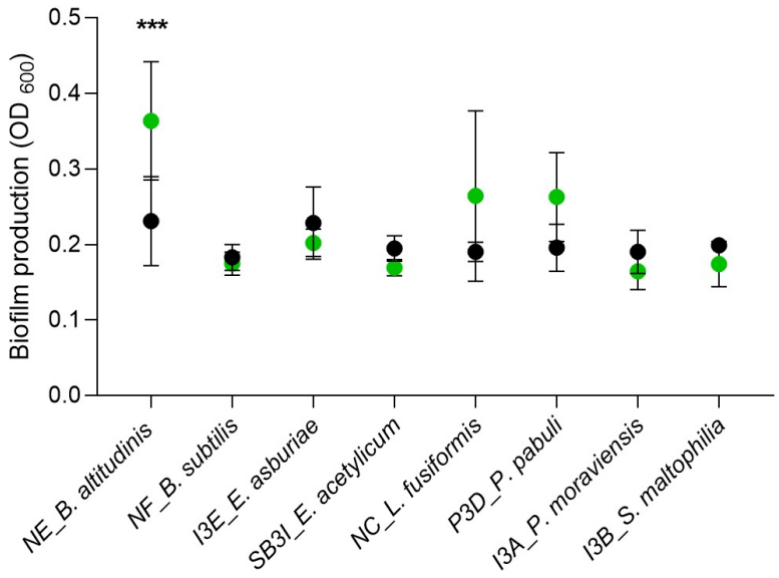

Figure 14. Biofilm production screening analysis

The box plot graph represents the biofilm production of eight strains under control conditions (black circle) and salt conditions at 0,5 mM (green circle). Each value is the mean of five replicates. Error bars represent ± standard deviation. The sets of data were compared by two-way ANOVA test and 95% confidence intervals (with Šidák's post-tests), where the asterisk represents a statistically significant difference at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****), respect to the values recorded in the control condition.

The majority of candidates showed no significant differences in biofilm formation under control or salt conditions. However, the candidate NE_*B. altitudinis* proved to be the one with the best results, having a significantly higher biofilm production under slat conditions. Nevertheless, NC_*L. fusiformis* and P3D_*P. pabuli* also demonstrated a tendency for positive biofilm production under salt conditions.

Auxins production

PGPB are known to produce auxins, hormones that stimulate root growth. Plants are able to secure and expand their accessible water and nutrient area thanks to auxins produced by PGPB (Park *et al.*, 2021). Moreover, this phytohormone is also behind the regulation of root architecture, which can be rearranged to cope with salt stress. Due to its importance, we analysed the capacity of our candidates for the production of auxins by growing them in LB with L-tryptophan.

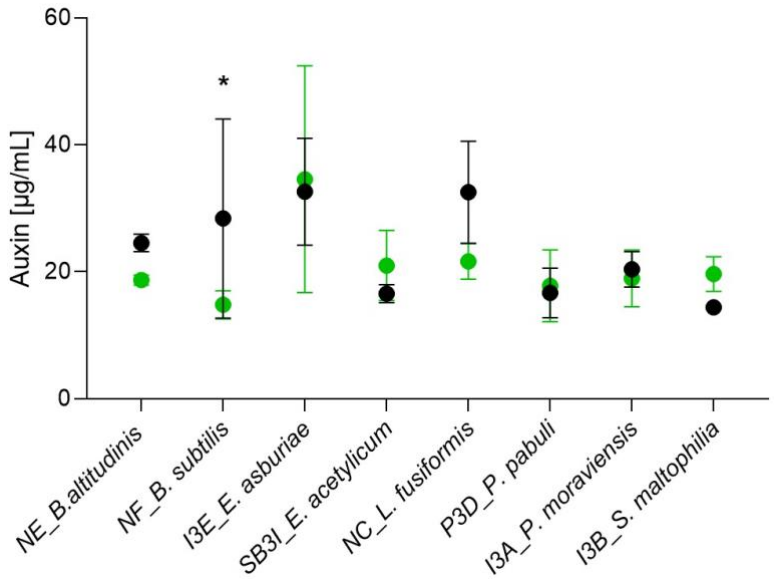

Figure 15. Auxins production screening analysis

The box plot graph represents the auxins production of eight strains under control conditions (black circle) and salt conditions at 0.5 mM (green circle). Each value is the mean of five replicates. Error bars represent ± standard deviation. The sets of data were compared by two-way ANOVA test and 95% confidence intervals (with Šidák's post-tests), where the asterisk represents a statistically significant difference at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****), respect to the values recorded in the control condition.

Overall, as can be seen in Fig. 15, the majority of the strains presented good results in auxin production (above 14 µg/mL), with no significant differences compared with the control. Although there were no outstanding candidates, the strains I3E_*E. asburiae*, SB3I_*E. acetylicum*, and I3B_*S. maltophilia* showed a positive tendency to surpass the control conditions under salt stress. Finally, the NF_*B. subtilis* strain showed a significant difference compared with the control, presenting lower auxin production under salt conditions (14.8).

ACC deaminase activity

ACC deaminase enzyme is one of the most important molecules produced by PGPB due to its impact on stress regulation by catalysing the stress hormone ethylene through cleavage of ACC (Orozco-Mosqueda, Glick and Santoyo, 2020). In this context, the candidates were grown in a medium using ACC as the sole N and C source.

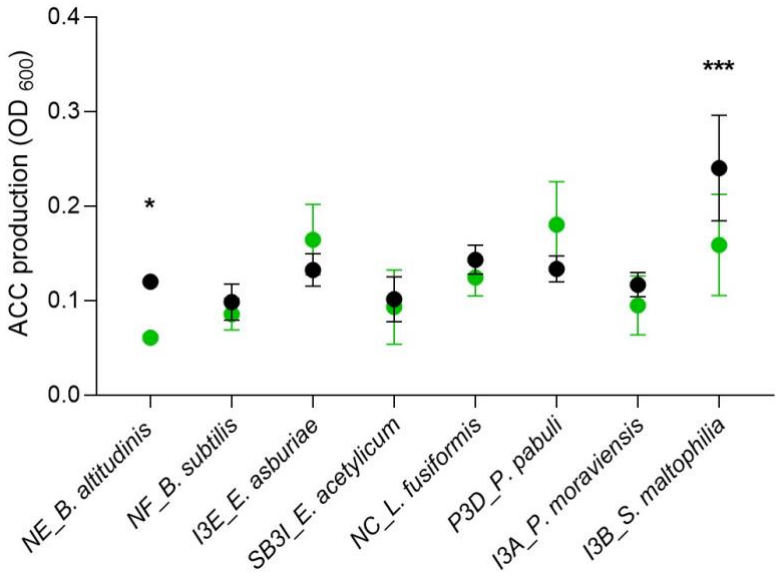

Figure 16. ACC deaminase production screening analysis

The box plot graph represents the growth recorded in ACC medium as sole nitrogen and carbon source (indicative of ACC deaminase production) of eight strains under control conditions (black circle) and salt conditions at 0,5 mM (green circle). Each value is the mean of five replicates. Error bars represent ± standard deviation. The sets of data were compared by two-way ANOVA test and 95% confidence intervals (with Šidák's post-tests), where the asterisk represents a statistically significant difference at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****), respect to the values recorded in the control condition.

A comparison of both conditions revealed that the majority of the candidates did not show any significant differences when compared to the control conditions (Figure 16). The two strains showed a positive tendency to produce this enzyme above average (I3E_*E. asburiae* and P3D_*P. pabuli*). On the other hand, one of the eight strains (I3B_*S. maltophilia*) showed a significant difference compared with the control, obtaining a lower value under salt conditions, which might be due to the fact that is also the strain that presents the highest value under control conditions.

Result Discussion

In order to select the best candidates for rice inoculation under saline stress, we first tested their capacity to grow under different salt concentrations (0, 0.5 M, 1 M and 2 M) (Figure 13). Our data indicate that, in general, all strains presented positive results under salt stress, indicating a potential for all of them as future inoculates. However, previous studies have shown that not all bacterial strains have the same ability to tolerate high salt concentrations (Ji, Tian, *et al.*, 2022). Therefore, identifying strains that are able to grow under high salt conditions is essential for successful inoculation in saline soils.

Two strains stood out in our study. NF_*B. subtilis* had the best growth results under 1 M salt concentration, with an OD of 0.68, while NE_*B. altitudinis* showed positive growth (0.25) under 2 M salt concentration. These results are in line with previous studies that have identified *B. subtilis* and *Bacillus spp.* as effective inoculants for promoting plant growth under saline stress (Ji, Chen, *et al.*, 2022; Ji, Tian, *et al.*, 2022). Moreover, our study found that I3E_*E. asburiae*, P3D_*P. pabuli*, and I3A_*P. moraviensis* showed increased growth compared to the control condition. This suggests that these strains may also be effective inoculants for saline soils.

In addition to growth, the ability to produce biofilms under saline stress is also important for bacterial inoculants. Biofilms help to protect bacteria from adverse environmental conditions and enhance their ability to interact with plant roots (Yin *et al.*, 2019). While the majority of our candidates showed no significant differences in biofilm production under control or salt conditions, NE_*B. altitudinis* had the best results, with significantly higher biofilm production under salt conditions. This is consistent with previous studies that have identified *Bacillus spp*. as effective biofilm-formers under saline stress (Ayaz *et al.*, 2022).

Auxin production is another important characteristic of bacterial inoculants, as auxins are important plant growth regulators that can help plants to cope with saline stress (Park *et al.*, 2021). In our study, the majority of the strains presented good results in auxin production (above 14 µg/mL) with no significant differences compared to the control. However, I3E_*E. asburiae*, SB3I_*E. acetylicum*, and I3B_*S. maltophilia* showed a positive tendency to surpass the control conditions under salt stress. This suggests that these strains may have potential as effective inoculants for promoting plant growth under saline stress.

Finally, ACC deaminase is an enzyme produced by some bacteria that can help plants to cope with saline stress by reducing the level of ethylene, a stress hormone that inhibits plant growth (Orozco-Mosqueda, Glick and Santoyo, 2020). In our study, the majority of the candidates did not show any significant differences in ACC deaminase production compared to the control condition. However, I3E_*E. asburiae* and P3D_*P. pabuli* showed a positive tendency to produce this enzyme above average. On the other hand, I3B_*S. maltophilia* showed a significant difference compared to the control, with lower ACC deaminase production under salt conditions. This may be due to the fact that I3B_*S. maltophilia* also had the highest ACC deaminase production under control conditions.

Chapter 3. Plant treatment: plant growth promotion and salt tolerance-enhancement

Soil salinity is a significant impediment to plant growth. However, recent research has shown that the application of ST-PGPB (Salt Tolerant Plant Growth-Promoting Bacteria) has the potential to overcome this problem (Kumar *et al.*, 2021). In this work, the growth of rice plants was tested in the presence and absence of salt stress, and with/without the individual application of four bacterial inoculums (P3D_Paenibacillus pabuli, NF_Bacillus subtilis, NE_Bacillus altitudinis, and NC_Lysinibacillus fusiformis). These bacterial strains were selected as the best candidates in previous experiments.

To conduct the experiment, a hydroponic system was designed. Here, the plants were treated with the bacterial inoculum and subjected to salinity conditions (EC= 12 dS/m) for 13 days. After 28 days, the plants were sampled for Chop-PCR analysis, and the phenotypical parameters were recorded. The results of the study should be considered tentative, as the system require adjustment- However, the individual application of these bacterial inoculums showed relevant insights in rice development under regular and salt stress conditions. Among them, the inoculated plants exhibited changes in root architecture under salt conditions compared to the non-inoculated plants. These findings are a promising initial step and their potential as plant growth-promoting bacteria and to alleviate the negative effects of salt stress.

Phenotypic analysis

As mentioned earlier, after 28 days, the plants were harvested and a phenotypic evaluation was performed. The phenotypic evaluation of the plants under control and salt conditions is presented in Fig. 17 and Fig. 18, respectively. Under regular conditions, the plants treated with the bacterial inoculums showed a normal phenotype with no major outlier compared to the mock plants. However, under salt stress conditions, the plants treated with NF_*B. subtilis* and NE_*B. altitudinis* were visually healthier. Moreover, the plants treated with P3D_*P. pabuli*, NF_*B. subtilis*, and NE_*B. altitudinis* showed a slight positive difference in height compared to the mock plants and even to the plants treated with NC_*L. fusiformis*. These visual differences were reflected in several quantified parameters such as shoot height, root length, and total/root dry weight (Figure 19).

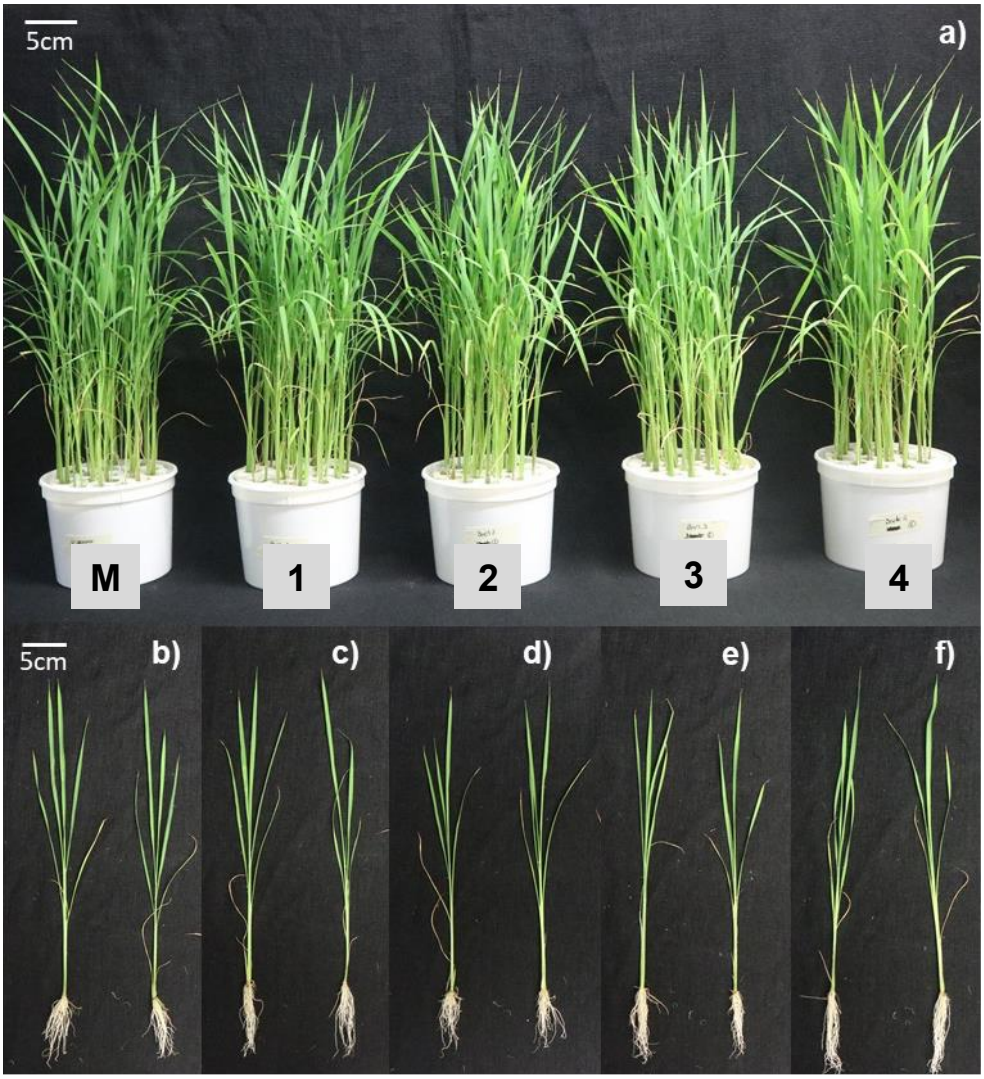

Figure 17. Growth and development of non-inoculated and inoculated rice plants under control conditions

The pictures represent the growth of the cv. Nipponbare in the hydroponic system (a). The full plant view of the representative plants can be seen in the images (b) – non-inoculated and plants inoculated with the strains (c) - P3D_*Paenibacillus pabuli,* (d) NF_*Bacillus subtilis,* (e) NE_*Bacillus altitudinis,* and (f) NC_*Lysinibacillus fusiformis,* (1,2,3,4) respectively, under no-salt conditions.

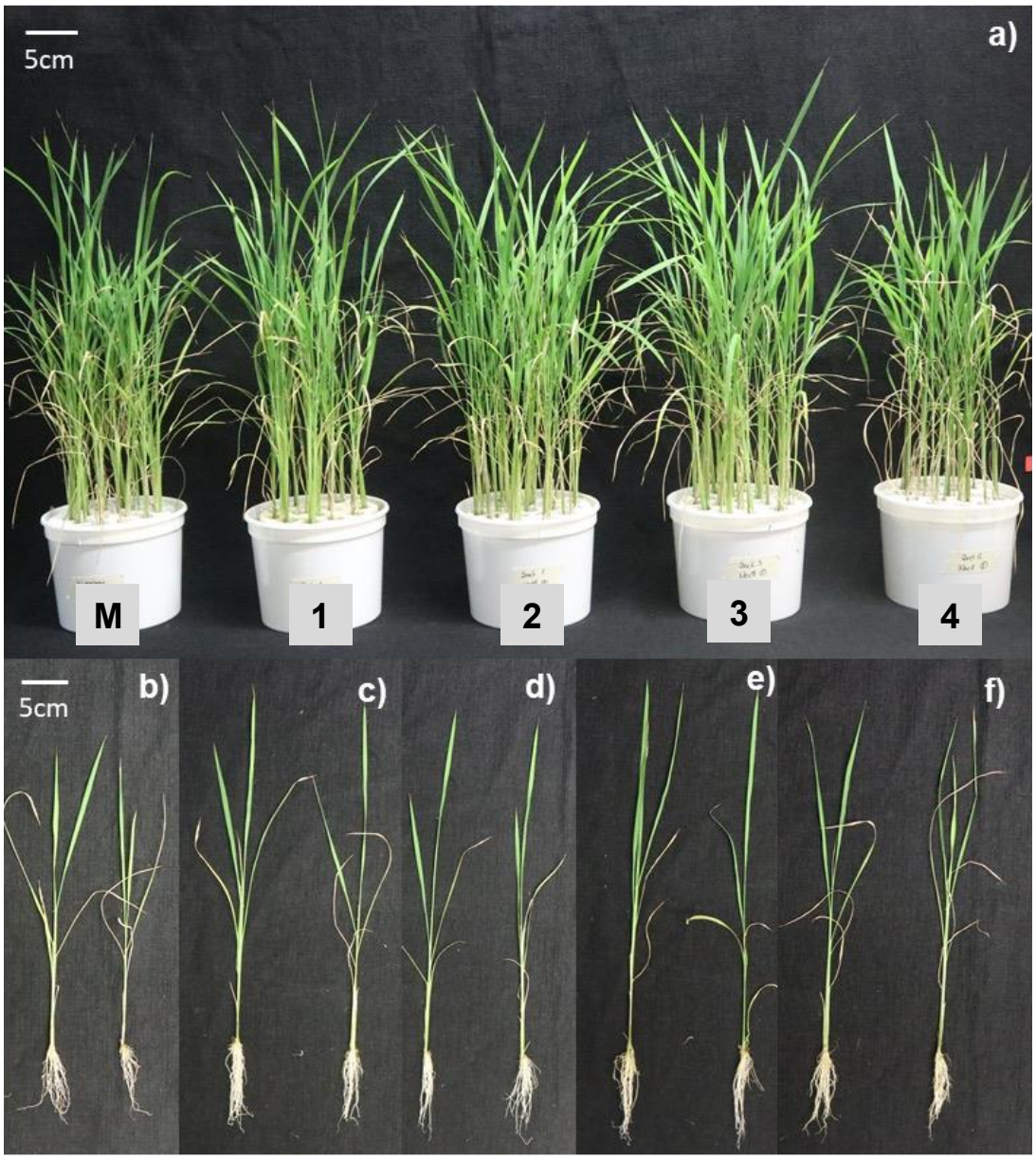

Figure 18. Growth and development of non-inoculated and inoculated rice plants under salt conditions

The pictures represent the growth of the cv. Nipponbare in the hydroponic system (a). The full plant view of the representative plants can be seen in the images (b) – non-inoculated and plants inoculated with the strains (c) - P3D_*Paenibacillus pabuli,* (d) NF_*Bacillus subtilis,* (e*)* NE_*Bacillus altitudinis,* and (f) NC_*Lysinibacillus fusiformis,* (1,2,3,4) respectively, under salt conditions.

Regarding shoot height (Figure 19a), there was a minimal significant difference between the mock and treated plants under regular conditions (no salt). However, considering the root length (Figure 19b), the treatment with the bacterial inoculums P3D_*Paenibacillus pabuli* and NC_*Lysinibacillus fusiformis* showed a positive significant difference compared to the mock plants, with an increase of 26,9% and 23,7%, respectively. These results were confirmed by the data in total dry weight (Figure 19c), where significant differences were visible in all bacterial inoculums, although less in NC_*Lysinibacillus*

fusiformis (1 - 43%, 2 – 36%, 3 – 47% and 4 – 20%), which was also reflected in the root dry weight (1 – 36%, 2 – 25%, 3 – 18% and 4 – 3%) (Figure 19d).

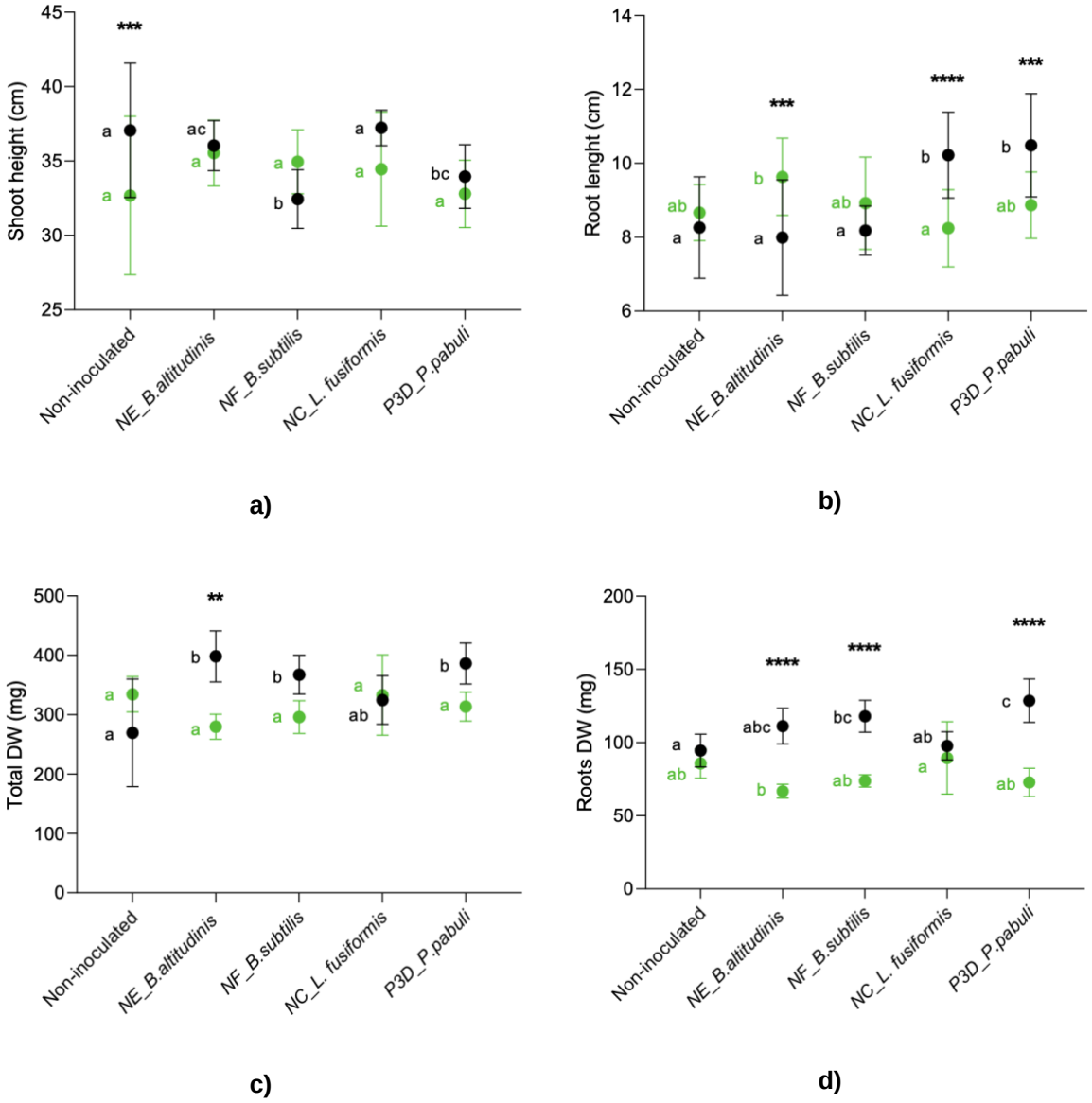

Figure 19. Phenotypic parameters of non-inoculated and inoculated rice plants under no-salt and salt conditions

The box plot graphs show the total dry weight (DW) (a), roots DW (b), shoot height (c) and root length (d) recorded for each treatment under control conditions (black circle) and salt conditions at 0,5 mM (green circle) (n=15). Error bars represent ± standard deviation. The sets of data were compared with two different data analysis. A two-way ANOVA test and 95% confidence intervals (with Šidák's post-tests), where the asterisk represents a statistically significant difference at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****), respect to the values recorded in the control condition. And a two-way ANOVA test and 95% confidence intervals (with Tukey post-tests), where different letters represent significant statistical differences.

In contrast, under salt conditions, there were no major significant differences in any of the parameters when comparing the mock plants with the plants treated with bacterial inoculums, even though the

plants treated with bacterial inoculums demonstrated a tendency for higher shoots. However, when comparing salt and control conditions, the results changed, particularly in root length and total/root dry weight. As expected, the mock plants showed a significant decrease in shoot height under salt conditions (12%), which did not occur in plants treated with bacterial inoculums. This might indicate that the plants treated with bacterial inoculums adapted better to the imposed salt stress. Moreover, the root dry weight seemed to have a significant decrease in plants treated with P3D_*Paenibacillus pabuli*, NF_*Bacillus subtilis*, and NE_*Bacillus altitudinis* (43%, 37% and 40%, respectively), which was also supported by the statistically significant decrease in root length in plants treated with P3D_*Paenibacillus pabuli* and NC_*Lysinibacillus fusiformis* (15% and 19%, respectively). These results might show a possible reaction triggered by the bacteria to help the plant cope and adapt better to the adverse salt conditions.

The results presented in the previous text demonstrate the potential of our candidate strains P3D_*Paenibacillus pabuli*, NF_*Bacillus subtilis*, NE_*Bacillus altitudinis*, and NC_*Lysinibacillus fusiformis* to improve plant growth under salt stress conditions in rice plants at different levels. This finding is in line with previous studies that have also reported the positive effects of these bacterial strains on plant growth and tolerance to abiotic stress.

For instance, a study by Lee et al., (2021) demonstrated that *P. pabuli* enhanced plant growth and nutrient uptake in wheat plants under salt stress conditions. Similarly, the application of *B. subtilis* has been shown to improve plant growth and tolerance to various abiotic stresses in different kind of plants (Lastochkina, 2020; Sagar *et al.*, 2022). Additionally, *B. altitudinis* has been reported to promote plant growth and enhance salt tolerance in rice plants (Jiao *et al.*, 2022), while *L. fusiformis* has been shown to improve drought tolerance in pepper plants (Passera *et al.*, 2021).

Under salt stress conditions, plants undergo changes in their root architecture as a survival strategy. One of the common responses to salinity stress is the reduction in root growth and branching, which can lead to a smaller root system (Hussain *et al.*, 2018). In addition to root growth and branching, plants may also undergo changes in root morphology under salt stress conditions, such as changes in root diameter and the formation of root hairs. These changes can help the plant increase its surface area for nutrient and water uptake, but they may also increase the plant's contact with toxic salts (Farooq *et al.*, 2022). Beneficial bacteria can also induce changes in root architecture under salt stress conditions as a survival strategy. For example, the application of Pseudomonas fluorescens has been shown to promote the development of longer and more branched roots in wheat plants under saline conditions (Fathalla and El-Mageed, 2020). Similarly, the inoculation of *Bacillus subtilis* has been reported to increase root length and surface area in rice plants under salt-stress conditions (Rekha *et al.*, 2018). These changes in root architecture can help plants to explore a greater volume of soil and access more water and nutrients, thereby improving their tolerance to salt stress. In our system, the contact with salt is very intense, with no protection in soil aggregates or other structures, so root system reduction mediated by bacteria may be a mechanism that helps to overcome the stressing conditions in the short term. Overall, the changes in root architecture under salt stress are a complex and dynamic process

that depends on various factors, including the plant species, the type and severity of salt stress, and the plant's overall response to stress.

Chop-PCR analysis

The Chop-PCR technique can provide semi-quantitative information on the methylation status of a particular genomic region in different samples, which can be useful in studying the epigenetic regulation of gene expression in plants (Ramalho-Carvalho, Henrique and Jerónimo, 2018). The main goal of this assay was to understand the hypothetical role of PGPB in inducing salt tolerance in salt-sensitive rice cultivars, and whether this reaction is also related to DNA methylation changes.

As mentioned above, Chop-PCR uses partial digestion by methylation-sensitive restriction enzymes to detect DNA methylation at specific loci. This technique involves extracting the plant's genomic DNA and digesting it with a methylation-sensitive restriction enzyme, which cuts only the unmethylated DNA at specific recognition sites. The resulting fragments are PCR-amplified using primers flanking the region of interest. Then, the PCR products are analyzed by gel electrophoresis, and the presence of the expected band size indicates the absence of methylation at the region of interest, while the absence of the band indicated the presence of methylation (Dasgupta and Chaudhuri, 2019). In general, the results of this technique are analyzed by the correlation between hyper-methylation to gene silencing and hypo-methylation to active transcription (Steward *et al.*, 2002). This Chop-PCR concept is illustrated in Fig. 20.

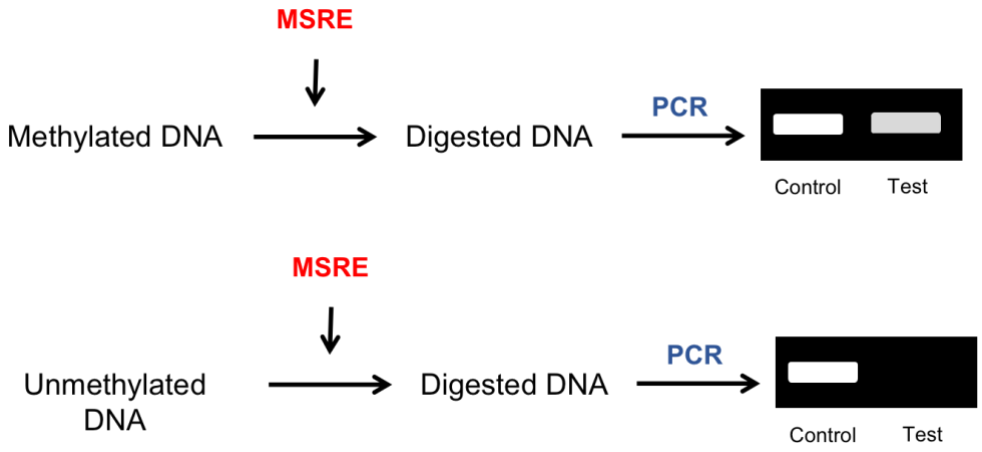

Figure 20. Principle of Chop-PCR.

(Source: Dasgupta and Shubho, 2019)

Hence, the sampling occurred five days before the finalization of the assay, when the phenotype was not yet happening. The samples where then prepared for Chop-PCR analysis by dividing shoots and roots. The results of the analysis were performed by comparing the set of plants treated with the four bacterial inoculants (P3D_*Paenibacillus pabuli*, NF_*Bacillus subtilis*, NE_*Bacillus altitudinis*, and NC_*Lysinibacillus fusiformis*), using 'Mock' and 'Pokkali' as control. Moreover, the analysis also considered the set of samples grown under salt and non-salt conditions. All raw data (electrophoresis gels) regarding enzyme digestion patterns and full-picture electrophoresis gels are presented in

Annexes 6 and 7 respectively. We need to indicate that this experiment was performed in a 'first-approach' context because of the lack of time for a more extensive analysis. This is the cause not all the controls appeared clearly in some cases.

The results recorded in the samples collected from the shoots of the plants are shown in Fig. 21. The data show that the genes Os05g38150 (G1) and Os03g17690 (G8) under all treatments are hyper-methylated, which is reflected by the presence of amplification of the bands, as in the control. On the contrary, the genes Os11g26790 (G5) and Os01g46970 (G6) present the total of the samples as hypo-methylated, which is reflected in the absence of bands. There are also genes for which the results are unclear due to the lack of repetitions, optimization, or errors in execution. Despite repetitions and result confirmation, the present results are worth mentioning. For example, in the case of gene Os07g47100 (G3), when the plants were treated with the strain NF_*B. subtilis*, the gene was hypo-methylated under salt conditions (no band or fainting). Some other genes showed a band fainting, so we would need to repeat and optimize the method to be sure of their actual performance. The relevance of these genes in the interaction process under salt stress could be relevant, but by now we cannot confirm their influence on the interaction.

However, fortunately, some genes presented clear results. Hence, the plants treated with strain NC_*L. fusiformis* seem to induce hypo-methylation of the gene Os06g48810 (G2) under salt conditions, which can also be observed as well in Pokkali plants without bacteria treatment. The main function of this gene is related to membrane transport, acting as a Na^+/K^+ symporter (Islam *et al.*, 2016), which may indicate to be regulated by the interaction with this strain as part of the salt defense mechanism of the rice plant. This result may indicate that, under this interaction, the gene is epigenetically regulated, diminishing the methylation level, which could induce overexpression of the gene. On the other hand, in plants treated with the strain P3D_*P. pabuli*, the Os02g02400 (G7) gene was hyper-methylated under salt conditions. This gene is active in combating the aversive effects of hydrogen peroxide through the synthesis of the powerful antioxidant metalloenzyme catalase isozyme A (Jung *et al.*, 2021). These results might indicate that strain P3D_*P. pabuli* is able to regulate ROS-scavenging system of the plant. This could be due to the bacterial production of antioxidants that regulate the plant production, which would allow the plant to focus on other mechanisms to overcome salinity stress. As shown previously, this strain maintains ACC deaminase activity under salt conditions controlling ethylene, which plays an important role in ROS accumulation under stress. Despite these partial clues, is still very soon to elucidate why or how is this regulation happening, and more experiments are needed to fully understand this mechanism.

This analysis was also performed by using DNA from plant roots. As shown in Fig.22, the genes G1, G8, G5, and G6 indicate the same results as before: hyper-methylation and hypo-methylation, respectively, in all the samples. Interestingly, G2 showed the same results as in the shoot samples, with the addition of hypo-methylation in plants treated with NE_*B. altitudinis*, although less conclusive when compared with the soot samples. Furthermore, the gene Os12g44360 (G4), even though there were no differences in the shoot samples, here it was hypo-methylated in the roots of plants treated with P3D_*P. pabuli* under control conditions and with NC_*L. fusiformis* under salt conditions. Moreover,

gene G7 also showed the same results in roots as it did in shoot samples, where hyper-methylation was observed under salt conditions in plants treated with P3D_*P. pabuli*. In this case, there was a more evident result, which also resulted in hyper-methylation in the Pokkali plants. As mentioned above, this gene is important for combating the adverse effects of hydrogen peroxide. In this case, it even shows the same behaviour that was observed in the Pokkali plants. Due to their salt-tolerance capacity, this might be a very good indicator of salt tolerance, as indicated by the selected candidates for this study (P3D_P. pabuli).

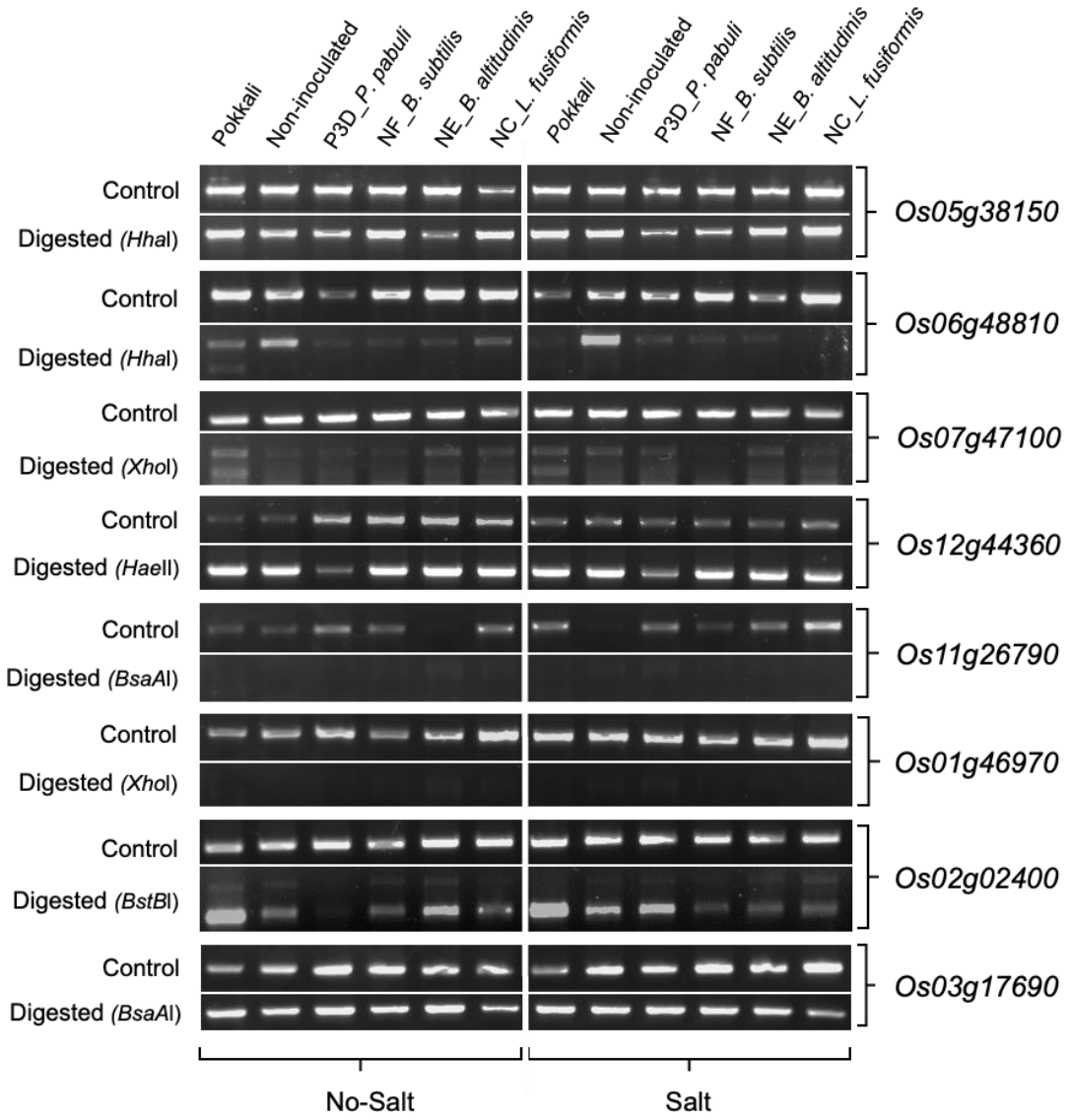

Figure 21. Chop-PCR analysis of shoot samples under no-salt and salt conditions

Chop-PCR analysis of DNA methylation at a group of genes related to salt stress tolerance in rice. The Methylation-Sensitive Restriction Enzymes, *BsaA*I, *BstB*I, *Hae*II, *Hha*I and *Xho*I) are indicated on the left. The analyzed loci are indicated on the right. The treatment description is indicated at the top and the bottom of the figure (Bacterial inoculum and salt, respectively).

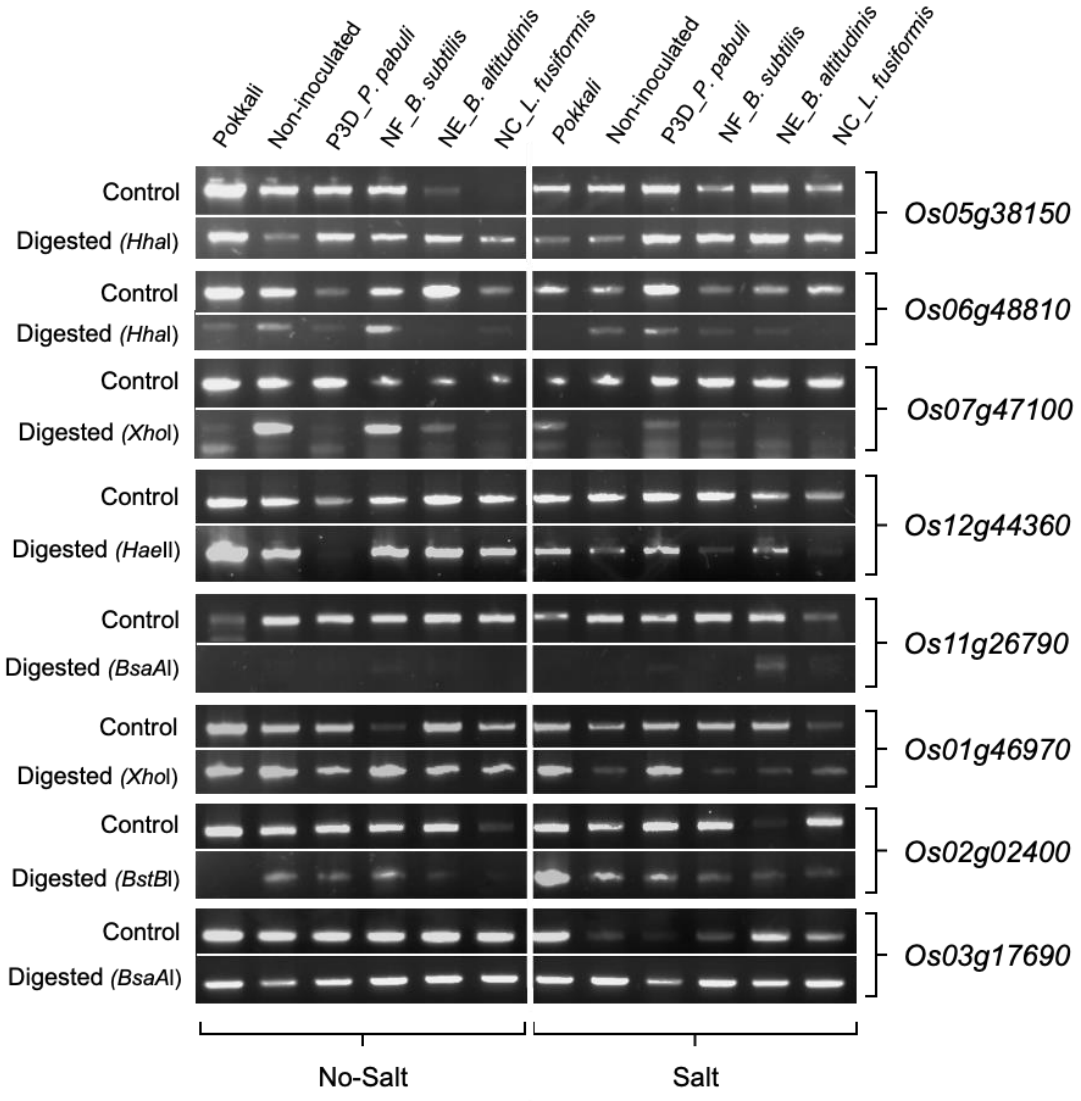

Figure 22. Chop-PCR analysis of root samples under no-salt and salt conditions

Chop-PCR analysis of DNA methylation at a group of genes related with salt stress tolerance in rice. The Methylation-Sensitive Restriction Enzymes, *Bsa*AI, *Bst*BI, *Hae*II, *Hha*I and *Xho*I) are indicted on the left. The analyzed loci are indicated on the right. The treatment description is indicated at the top and the bottom of the figure (Bacterial inoculum and salt, respectively).

Regarding all the results and the fact that this is a first-approach analysis performed in a very short period of time, we are aware that the optimization was not perfect and that the repetition and confirmation of all these data and the continuity of the experiments would be necessary. We are also aware of the disadvantages of using hydroponics as our main system because of the strong impact on the plants under salt stress and the possibility and difficulty of colonization by the bacteria. Therefore, tests as the colonization rate of these strains under normal and saline conditions are imperative for validation of the results, as well as the assessment and adjustment of the hydroponic system. However, this first approach already showed a very interesting set of results to consider in future lines. While there is still limited research on the potential influence of plant growth-promoting bacteria (PGPB) in

inducing DNA methylation changes during salinity stress, this is an area of growing interest. A recent study by Hosseinpour et al., (2022) is one of the few examples of articles investigating this topic. The study found that wheat plants (*Triticum aestivum* L.) exposed to salt stress (250 mM NaCl), and treated with copper (II) oxide nanoparticles and *Bacillus subtilis*, *Lactobacillus casei* and *Bacillus pumilis* , showed an effect of DNA hypo-methylation in all treatments, which seems to play a protective role under saline stress conditions.

Conclusions and Future Perspectives

The rising levels of salinity are becoming a significant problem for agricultural productivity (Shrivastava and Kumar, 2015). However, the use of both seed-borne and endophytic microbiomes originating from the microbiota of halophytic plants may have a beneficial effect in reducing the impact of salinity stress, ultimately leading to sustainable improvements in agricultural productivity (Kumar *et al.*, 2020).

As main conclusions, our study revealed that:

- Even though from the same species, different cultivars recruited a different set of microbial communities indicating the plant's effect on microbiota shaping and modulating microbial interactions in the rhizosphere through the use of recruiting compounds. Moreover this could be behind the differential seed-borne microbiota population patterns in different cultivars detected in this work. This highlights the importance of considering cultivar-specific microbiota in future sustainable agricultural practices;

- Strains isolated from the seed-borne and endophytic microbiota of the rice salt tolerant cultivar Pokkali (P3D_*Paenibacillus pabuli,* NF_*Bacillus subtilis,* NE_*Bacillus altitudinis,* NC_*Lysinibacillus fusiformis)* showed a remarkable potential to be used as future biostimulants in sustainable agriculture as plant growth-promoting bacteria; moreover, NF_*Bacillus subtilis,* NE_*Bacillus altitudinis*, and even maybe P3D_*Paenibacillus pabuli*, can be proposed as salt tolerance-enhancers after the results we obtained;

- Under a hydroponic system, salt-sensitive rice plants appear to reveal positive tendencies regarding salt-tolerance improvement when inoculated with mentioned strains. This phenotypic analysis at different levels showed promising results regarding plant growth promotion under salt stress conditions;

Moreover, we were able to discern some promising traits in epigenetic regulation during salt tolerance enhancement mediated by bacteria in rice plants. Here, the genes OsHKT2;1 and OsCATA, being these related to membrane transport and the combat of aversive effects of ROS, showed promising results. This is the first report of an epigenetic trait in the context of rice, salt, and bacterial interactions. Therefore, this research line could provide interesting outputs and insights after careful adjustment of the protocols and incorporation of approaches that provide more resolution.

Finally, we emphasize the potential use of these PGPRs as bioinoculants in agriculture that involves saline soil. The variety of PGPRs with various PGP traits found in the seeds and rhizosphere of halophytic plants is a valuable asset for enhancing crop resistance to salinity and advancing agriculture that involves saline soil in the future.

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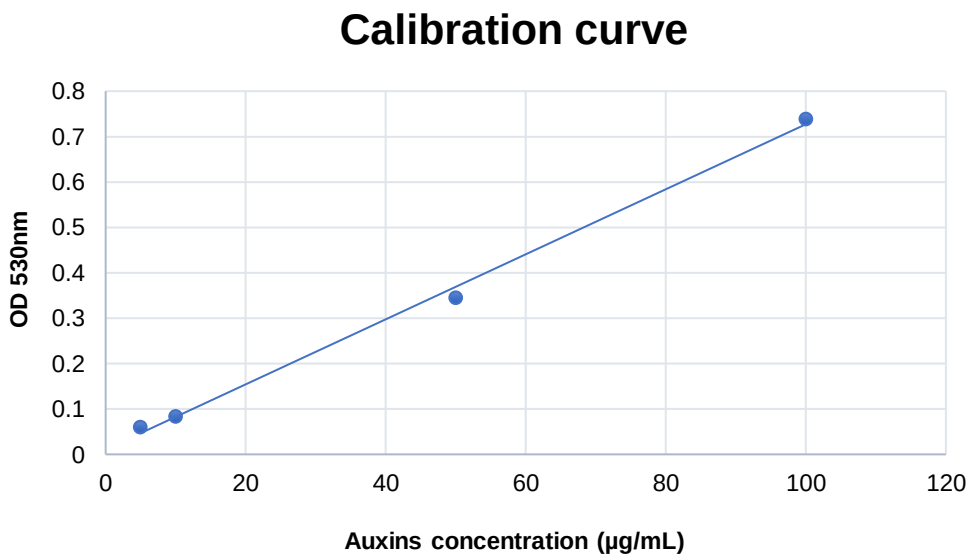
Annex

Annex 1 - Calibration curve for auxin quantification [µg/mL]



Annex 2 – Minimal medium M9 Composition (Stock solutions)

M9 salt solution (10X)	Na_2HPO_4-$2H_2O$	75.2 g/L	
	KH_2PO_4	30 g/L	
	NaCl	5 g/L	
	NH_4Cl	5 g/L	
100X trace elements solution	EDTA	5 g /L	13.4 mM
	$FeCl_3$-$6H_2O$	0.83 g/L	3.1 mM
	$ZnCl_2$	84 mg/L	0.62 mM
	$CuCl_2$-$2H_2O$	13 mg/L	76 µM
	$CoCl_2$-$2H_2O$	10 mg/L	42 µM
	H3BO3	10 mg/L	162 µM
	$MnCl_2$-$4H_2O$	1.6 mg/L	8.1 µM

Annex 3 – Yoshida's solution Composition

Concentration and type of chemical compounds. Adapted from (Almeida et al 2016).

ELEMENT	REAGENT	YOSHIDA MEDIUM (g/L)
Macronutrient (five independent solutions)		
N	Ammonium nitrate (NH_4NO_3)	91.40
P	Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)	35.60
K	Potassium sulfate (K_2SO_4)	71.40
CA	Calcium chloride, dihydrate ($CaCl_2 \cdot 2H_2O$)	117.35
MG	Magnesium sulfate, 7-hydrate ($MgSO_4 \cdot 7H_2O$)	324.00
Micronutrient (one solution)		
MN	Manganese chloride, 4-hydrate ($MnCl_3 \cdot 4H_2O$)	1.500
MO	Ammonium molybdate, 4-hydrate [$(NH_4)6Mo_{24} \cdot 4H_2O$)]	0.074
ZN	Zinc sulfate, 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	0.035
B	Boric acid (H_3BO_3)	0.934
CU	Copper sulfate, 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.031
FE	Ferric chloride, 6-hydrate ($FeCl_3 \cdot 6H_2O$)	7.700
	Citric acid, monohydrate ($C_6H_8O_7 \cdot H_2O$)	11.900

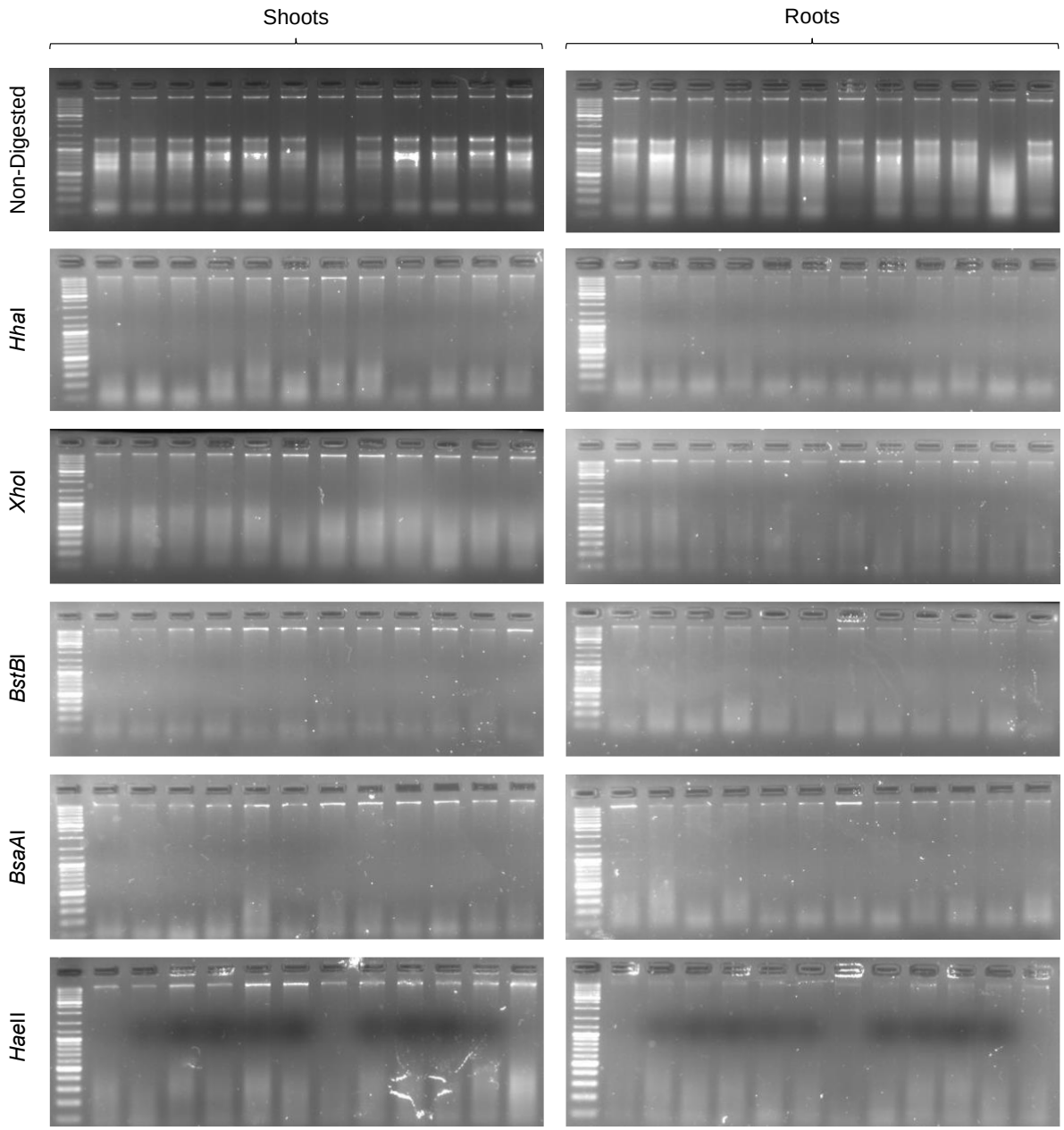
Annex 4 – Enzyme specifications

Enzyme	Incubation °C	Inactivation °C	Buffer
*Hha*I	37	65	Cut smart
*Xho*I	37	65	NEB 4
*BstB*I	65	-	NEB 4
*BsaA*I	37	-	Ppu21
*Hae*II	37	80	Cut smart

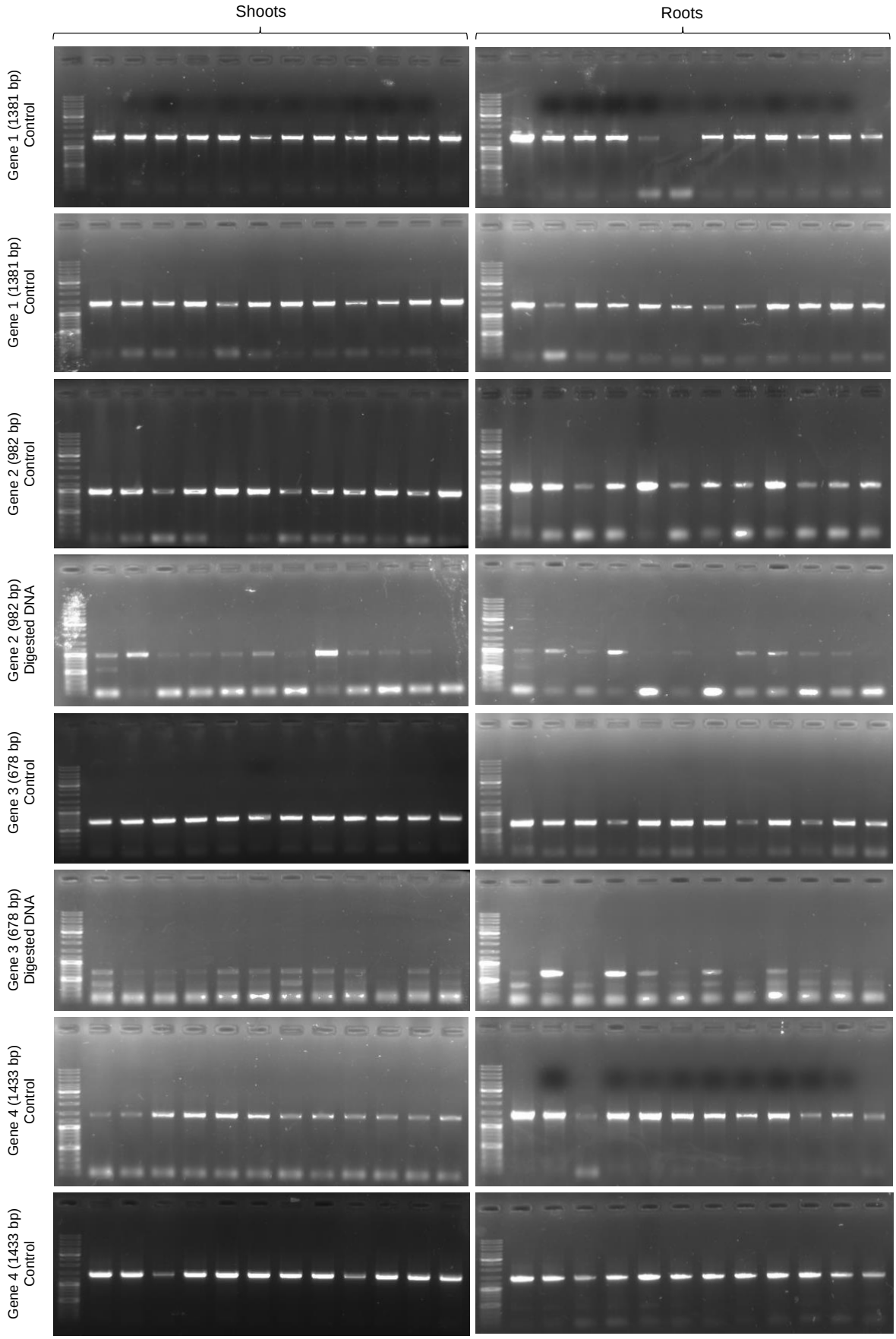
Annex 5 – Gene and primer specifications

Label	Gene	LOC	5'-3'	3'-5'	Annealing (°C)
O1	OsP5CS	Os05g38150	TGCCCAGTGGAGT TACACAC	ATTGCAGGCTGCTGG GTAAT	57.8
O2	OsHKT2;1	Os06g48810	GCTTCGGAAGGAT CGGTAGA	GAATTCCAGTCGACA GCACC	58
O3	OsNHX1	Os07g47100	AGAGGGGAGTGGA TTGGTTG	ATCGACGGACAGAC AGCTAG	59.3
O4	OsSOS1	Os12g44360	GGAACGAACCTCT CCCTTGA	GTCATGTAGCTGCCG TCTTG	59.7
O5	OsRab16A	Os11g26790	GATGGAATGGGAG GGAGGAG	CAATTTCCGGCCGTT GATCT	60.5
O6	OSBZ8	Os01g46970	CACCTCACCAAACA CCACTG	GTAACATCAGTGCTG GCTCG	59.2
O7	OsCATA	Os02g02400	GAGCCTCCACACC TTCTTCT	GAGTAGTAGATCCCC GGCAC	58.7
O9	OsAPX1	Os03g17690	CACACCCTGGTTAG TTTGGC	GTAACATCAGTGCTG GCTCG	58.6

Annex 6 – Enzyme digestion patterns and full-picture electrophoresis gels



Annex 7a –Full-picture electrophoresis gels (Genes 1- 4)



Annex 7b –Full-picture electrophoresis gels (Genes 5- 8)