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**Colonization pattern of rice introgression lines by *Kosakonia sacchari* RCA25 and associated drought-responsive gene expression**

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## **DEDICATION**

This entire project is dedicated to my family for their unwavering support. Thank you for the support. Most importantly to the Lord God All Mighty, for without him none of this could have ever been achieved.

## ABSTRACT

Rice (*Oryza sativa* L.) is increasingly threatened by drought and other climate-induced abiotic stresses, while overreliance on synthetic fertilisers raises growing environmental concerns. Beneficial endophytic bacteria, such as *Kosakonia sacchari* RCA25 offer a promising sustainable alternative; however, the influence of host genotype on endophytic recruitment and the molecular mechanisms underlying plant-endophyte interactions under stress remain poorly understood. This thesis aimed to characterise genetic variation in *K. sacchari* RCA25 root colonisation across a Vialone Nano × *Oryza rufipogon* introgression line (IL) population, assess genotype-dependent physiological responses to combined inoculation and drought stress, and establish a high-quality RNA sample collection for downstream transcriptomic analysis.

A colonisation screen was performed on 41 ILs and the two parental lines Vialone Nano and *O.rufipogon* under controlled conditions, with root colonisation quantified by colony-forming unit (CFU) analysis following surface root sterilisation, homogenisation, serial dilution, and plating on selective medium. A drought stress experiment was subsequently conducted on four selected genotypes (IL273, IL189, Vialone Nano and *O.rufipogon*), measuring shoot length, relative water content (RWC), and root colonisation under well-watered and water-stressed conditions with and without RCA25 inoculation. Total RNA was extracted from root tissue across 48 samples using a TRIzol – based protocol, and RNA integrity was assessed by agarose gel electrophoresis, Qubit fluorometric quantification, and Agilent 2100 Bioanalyzer capillary electrophoresis.

The colonisation screen revealed substantial variation across the IL panel with mean  $\log_{10}$  CFU/g values ranging from 6.07 to 8.17. IL36 and IL28 recording the highest colonisation levels IL6 and IL8 recording the lowest. *O.rufipogon* achieved significantly higher colonisation (8.23  $\log_{10}$  CFU/g) than Vialone Nano (7.29  $\log_{10}$  CFU/g) confirming that domestication has progressively reduced the capacity of cultivated rice to recruit beneficial endophytes (H1 and H2 supported). A three-way General Linear Model (GLM) revealed significant effects of Genotype ( $p < 0.001$ ), Treatment ( $p < 0.001$ ) and a Genotype × Treatment interaction ( $p = 0.007$ ) on shoot length; across all four genotypes, uninoculated controls consistently achieved greater shoot elongation than inoculated plants. However, no statistically significant inoculation effect on RWC was detected across genotypes, indicating that *K. sacchari* RCA25 did not confer consistent physiological benefits under the conditions tested (H3 not supported). IL samples consistently produced high-integrity RNA with 28S and 18S ribosomal bands in the expected 2:1 ratio. Batch 1 parental lines samples failed quality control with RIN values below 7; following protocol optimisation, Batch 2 samples showed marked improvements with many samples achieving  $RIN \geq 7$  and all samples exceeding the minimum concentrations threshold of 20ng/ $\mu$ L. All quality-confirmed samples have been submitted for RNA sequencing at IGA laboratory (Udine, Italy); sequencing is currently underway and assessment of H4 (genotype-dependent gene expression in response to colonisation) will be addressed upon receipt of the data.

These findings confirm that host genetic background is the primary determinant of *K. sacchari* RCA25 colonisation in rice, and the phenotypic colonisation and genomic data generated provide a foundation for future QTL mapping to identify candidate gene governing endophytic recruitment and drought resilience in rice.

## **ACRONYMS**

**ACC** : 1-Aminocyclopropane-1-Carboxylate

**ANOVA**: Analysis of Variance

**BC<sub>3</sub>SF<sub>5</sub>**: Backcross Self Fertilisation 5 generation

**CDS**: Coding DNA Sequencing

**CFU**: Colony-Forming Unit

**CV**: Coefficient of Variation

**DESeq2**: Differential Expression analysis

**DNase** :Deoxyribonuclease

**EPS**: Exopolysaccharides

**FC**: Field Capacity

**GLM**: General Linear Model

**HSD**: Honestly, Significant Differences (Tukey's)

**IAA**: Indole-3-Acetic Acid

**IL/ILs**: Introgression Line(s)

**LB**: Lysogeny Broth

**MDA**: Malondialdehyde

**NGS**: Next Generation Sequencing

**PCR**: Polymerase Chain Reaction

**PGPB**: Plant Growth-Promoting Bacteria

**QTL**: Quantitative Trait Locus

**RIN RNA**: Integrity Number

**RNA-seq**: RNA sequencing

**ROS**: Reactive Oxygen Species

**rRNA**: Ribosomal RNA

**RWC**: Relative Water Content

**SD**: Standard Deviation

**SynCom**: Synthetic Microbial Community

**UTR**: Untranslated Region

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## **INTRODUCTION:**

### *Enhancing Rice Climate Resilience through Plant-Microbiome Interactions*

#### **1.0 Overview of Rice Plant-Microbiome Interactions**

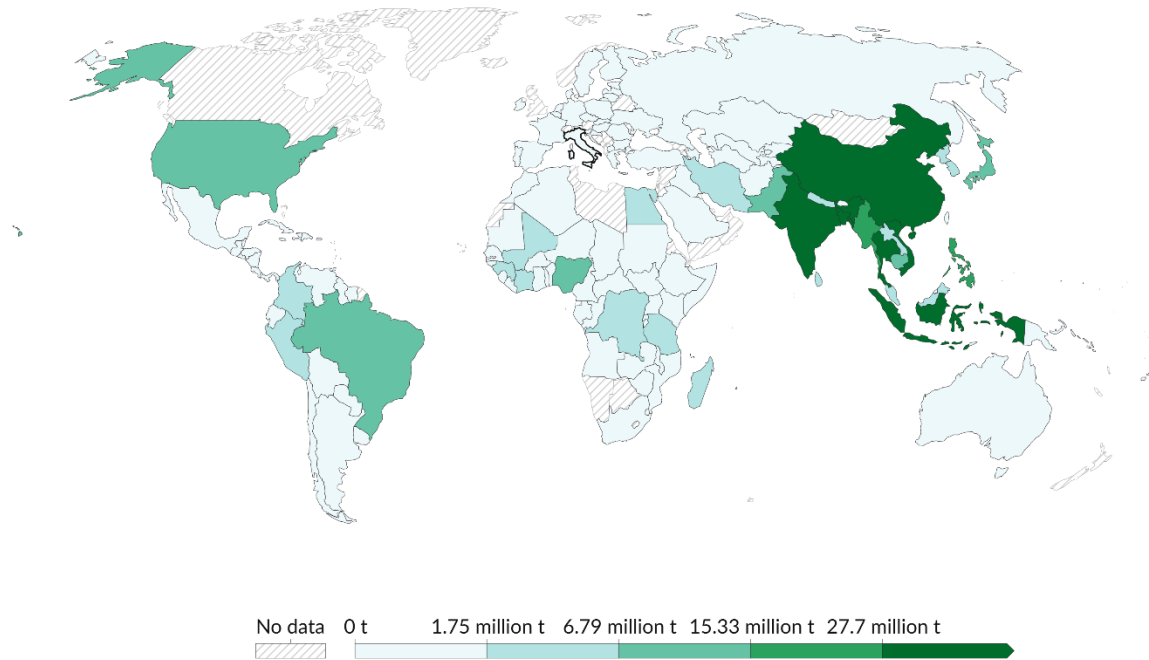
##### **1.1 The Role of Rice-Microbiome Studies in Ensuring Global Food Security**

Rice (*Oryza sativa L*) serves as the primary staple food for over half of the world's population, particularly in Asia, Africa and Latin America (Mohidem et al., 2022). According to (FAO, 2025), global rice production exceeds 520 million tonnes annually, with approximately 3.5 billion people depending on rice for more than 20% of their daily caloric intake. Global patterns of rice output in 2023 (Figure 1) illustrate the crop concentration in Asia, particularly in China and India, where the darkest green shading indicates national production exceeding 27.7 million tonnes.

Driven by continuous population growth – expected to approach 10 billion by 2050 (Farooq et al., 2023), rice output has risen through advances in plant breeding and agronomic management. Yet this progress is threatened, the Intergovernmental Panel on Climate Change (IPCC, 2023) projects that climate change induced drought alone could slash yield by 20-40% in key rice growing regions within the next 25 years, while floods, salinity intrusion, and emerging pests add further pressure (Algarni et al., 2025).

Traditional intensification relying on high doses of chemical fertiliser has underpinned past gains, but such inputs are increasingly expensive, energy-intensive and environmentally damaging, causing water pollution, diversity loss and soil degradation (Wang et al., 2020). Consequently, sustainable alternatives are urgently needed. Exploring beneficial plant-microbiome interactions offers an ecologically sound route to maintain productivity under stress (Kumar et al., 2022). Although microbial inoculants are now promoted as drought mitigation tools (Singh et al., 2020), most experiments test a single host genotype, thus overlooking genetic variation in microbial receptivity.

## Rice production, 2023



Data source: Food and Agriculture Organization of the United Nations (2025)

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*Figure 1 rice production by country in 2023. Source: Food and Agriculture Organisation of the United Nations (2025), reproduced under [CC-BY 4.0] (<https://creativecommons.org/licenses/by/4.0/>) via Our World in Data (Ritchie et al., 2023)*

### 1.1.2 Rice Holobiont: The foundation of the interaction between rice and microorganisms

The theoretical framework for comprehending the beneficial interactions between rice and soil microorganisms is explained by the “holobiont” concept, which characterises a host organism alongside its inherent microbial consortium as a singular ecological entity (Lyu et al., 2021). Within the framework of rice, the “rice holobiont” encapsulates the plant and the diverse group of microorganisms, including bacteria, archaea, fungi, and viruses that reside within different compartments: the rhizosphere (bacteria surrounding the roots), the rhizoplane (bacteria around the root surface), the endosphere (bacteria in internal tissues) and the phyllosphere (bacteria on the above-ground surfaces) (Chang et al., 2025a). The holobiont framework is crucial because it recognises that plants phenotypes result not solely from plant genetics but from the integrated

genetic potential of the host and its associated microbiome. This perspective shifts breeding strategies from focusing exclusively on plant genetics.

These microbial communities are not merely passive occupants; they engage in active interactions with the plants, thereby affecting their growth, development, nutrient assimilation, stress reliance, and resistance to diseases (Mukherjee et al., 2024). Rice plants secrete exudates—a complex mixture of organic compounds that attract and sustain specific microbial taxa (Chen and Liu, 2024). Beneficial microorganisms, in turn, influence plant physiology through mechanisms such as phytohormone synthesis, nutrient solubilisation, and the induction of systemic resistance, especially under adverse environmental conditions (Koza et al., 2022).

A critical goal of current agricultural research is to decipher this complicated communication to discover novel microbiome-based approaches for increasing crop yield and resilience (Zhao et al., 2024). A variety of factors influence the makeup and functionality of the rice microbiome, including plant genotypes, developmental phases, soil types, agricultural techniques, and environmental variables (Wang et al., 2020). This selective enrichment indicates that plants actively recruit and filter microorganisms from the soil (Huang et al., 2023). Similarly, studies have identified specific bacterial genera such as *Methylobacterium*, *Actinobacterium*, *Bulkhilus*, *Exiguobacterium*, and *Bacillus* in rice shoots and roots, which have a major impact on global carbon and nitrogen cycles at the ecosystem level (Wang et al., 2020). With the rice holobiont established as a foundational unit for plant performance, it is essential to examine how climate-driven abiotic and biotic stresses disrupt these interactions and underscore the microbiome's role in resilience.

### **1.1.3 Rice: Domestication, Genetics and Nutritional Value**

Rice was first domesticated from its wild progenitor, *Oryza rufipogon*, in the southern part of China approximately 10000 years ago (Fuller et al., 2010). Over the years, farmers applied continuous selection pressure for traits advantageous to agriculture, which include non-shattering seed disposal, large grain size, reduced seed dormancy and increased yields. This domestication process gave rise to the two major subspecies of *Oryza sativa*, *indica* (adapted to tropical and subtropical regions) and *japonica* (adapted to temperate climates), which exhibit substantial genomic and phenotypic divergence. Evidence indicates that a single domestication event of Asian

rice followed by subsequent diversification led to distinct genetic lineages adapted to different agroecological zones (Molina et al., 2011; Vaughan et al., 2008).

Rice is a diploid species with 12 pairs of chromosomes ( $2n = 24$ ) and a compact genome of approximately 380-430 million base pairs, making it the first crop plant genome to be fully sequenced (Sasaki and Burr, 2000). Several key genes that control agronomically important traits have been identified; so far, as an example the *Wx* (waxy) gene on the chromosome 6, which encodes granule-bound starch synthase and controls amylose content, a major determinant of grain texture and cooking quality, and the *sd1* (semidwarf 1) gene on chromosome 1, whose loss of function allele confers the semidwarf plant stature, fundamental for the so called green revolution varieties (Sano, 1984; Sasaki et al., 2002). Modern rice breeding strategies use DNA marker assisted selection (MAS), quantitative trait locus (QTL) mapping, and genome wide association studies (GWAS) to improve complex qualitative traits such as yield, stress tolerance, and grain quality.

Rice is high in carbohydrates; it provides about 28 g of starch, 2.0-2.7 g of protein and less than 0.5g of fat per 100g cooked (Vici et al., 2021). White rice has a lower protein and fibre compared to brown rice. White (polished) rice, produced by removing the bran and germ, is highly digestible and typically enriched with B vitamins and iron, it contains small amount of fibre (around 0.3-0.5 g/100g)(Farooq and Yu, 2025). White rice has a high glycemic index, leading to a faster blood glucose rise. In contrast, brown rice has the bran and germ, supplying roughly 1.5-2g fibre per 100g (Ravichanthiran et al., 2018) and has higher levels of magnesium, phosphorus, manganese and zinc, which are essential for better satiety, glycemic control and cardiometabolic health (Farooq and Yu, 2025). White rice is the main form of rice consumed globally, it has low iron, zinc and provitamin A, while it provides 60-80% of daily calories for many people around the world there is a high risk of micronutrient deficiencies, this has led to biofortification programs to increase Fe, Zn and provitamin A content (Kamruzzaman et al., 2025; Majumder et al., 2019; Senguttuvel et al., 2023a).

## 1.2. Impact of Climate Change on Rice Production and the Role of Microbiomes

### 1.2.1 Abiotic and Biotic Stresses Affecting Rice Cultivation

Rice cultivation is increasingly threatened by a multitude of abiotic and biotic stresses, many of which are worsened by climate change. Drought, salinity, extreme temperatures (heat and cold), flooding, soil acidity, sodicity, and nutrient deficits or toxicities are all examples of abiotic stresses that can significantly limit rice growth and yield (Misu et al., 2025). Drought is a major limitation in rain-fed rice habitats, impacting plant growth, development, and grain yield.

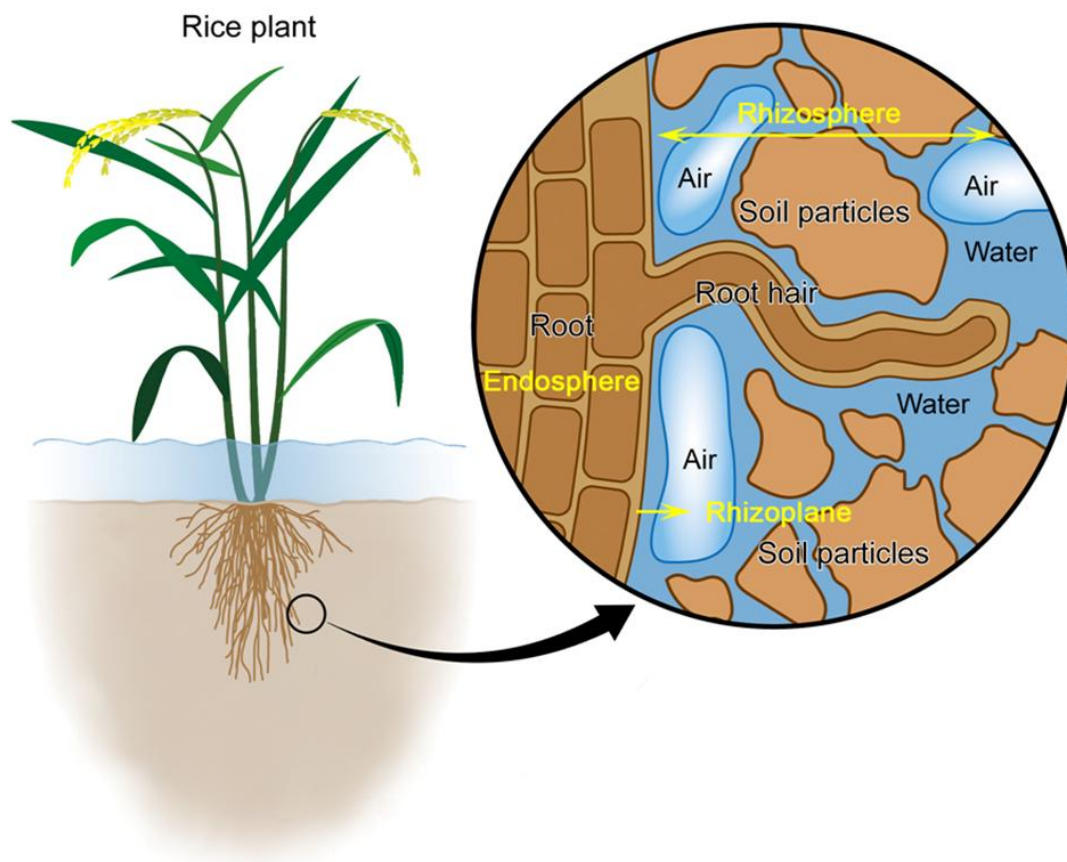


Figure 2 Rice root cross-section schematic: rhizosphere, rhizoplane, and endosphere in flooded soil. (Ding et al., 2019)

Salinity, especially in coastal regions and in areas with low irrigation water quality, can cause ion toxicity, osmotic stress, and decreased nutrient uptake, all of which have a negative influence on rice output (Dakshayini et al., 2025). Rising global temperatures threaten rice cultivation by disrupting critical stages including vegetative growth, reproductive stages such as pollination and spikelet fertility and grain filling, leading to reduced yields and compromised grain quality (Liu et al., 2021). Heat stress during the flowering stage is particularly detrimental, causing spikelet sterility and substantial yield losses in major rice-growing regions worldwide (Shrestha et al., 2022).

On the other hand, biotic stressors include a wide range of infections (fungi, bacteria, viruses) and pests (insects, nematodes) that can result in large output losses. Diseases such as rice blast (caused by *Magnaporthe oryzae*), bacterial leaf blight (produced by *Xanthomonas oryzae pv. oryzae*), and sheath blight (caused by *Rhizoctonia solani*) are common and severe in many rice-growing areas. The interplay of abiotic and biotic stresses can further complicate management efforts, as stressed plants are frequently more prone to disease attack.

The geographical distribution and intensity of these pressures are expected to fluctuate as climate change continues, providing new challenges to global rice production systems. Rising temperatures can attract new pests and viruses, while changing rainfall patterns might cause frequent and severe droughts or floods that harm rice crops (Skendžić et al., 2021). Soil salinisation is a rising concern, particularly in deltaic regions sensitive to sea-level rise and in dry and semi-arid zones where irrigation can contribute to salt accumulation, shaping the microbiome composition and root exudates (Afridi et al., 2024; Zambelli et al., 2025).

### **1.2.2 Microbiome-Mediated Mechanisms for Climate Resilience in Rice**

Beneficial rhizobacteria can modify gene expression in salt stressed rice, enhancing antioxidant enzyme activity and regulating osmotic substances (Sackey et al., 2025) it thus possible to improve plant tolerance to abiotic stresses, like drought, using beneficial root microorganisms, resulting in more sustainable and secure rice production (Misu et al., 2025). The plant-associated microbiome is critical for increasing rice tolerance to diverse climate-induced stresses, both abiotic and biotic (Munir et al., 2022). Beneficial bacteria use a variety of strategies to maintain plant health and productivity in adverse situations (Ullah et al., 2025). One of the keyways microorganisms help plants adapt to abiotic stress is by altering their physiological responses. For instance, under

drought stress, certain rhizobacteria can produce exopolysaccharides (EPS) that help in soil aggregation and moisture retention, or they can produce osmoprotectants (such as proline and trehalose) and phytohormones (like abscisic acid or ACC deaminase, which lowers ethylene levels) that help plants cope with water deficit (Munir et al., 2022). The mechanisms underlying microbiome-mediated climate resilience are discussed in section 1.8.3.

As shown above, the rice microbiome contributes to climate resilience through mechanisms such as antioxidants activation, osmoprotectants production, EPS-mediated moisture retention, and hormone regulation. However, domestication and intensive breeding may have unintentionally reduced the ability of modern cultivars to recruit and maintain these beneficial microbes. Therefore, a comparative analysis of wild and domesticated rice microbiomes is crucial to identify microbes functions that have been lost or weakened during domestication and could be reintroduced to enhance climate resilience (Section 1.3)

### **1.3 A comparative analysis of wild and domesticated rice microbiomes**

#### **1.3.1 Evidence for Microbiota Functional and Structural Variations**

Current research demonstrates that cultivated rice (*Oryza sativa*) varieties harbour less microbial diversity and a distinct microbial community than its wild counterpart, *Oryza rufipogon*. This difference reflects the consequence of plant domestication, intensive agricultural practises and crop variety selection (Kim et al., 2020). Modification of the rhizomicrobiome - the complex microbial communities inhabiting the rhizosphere and root tissues results in functional variations in nutrient cycling and plant-microbe associations. Consequently, cultivated rice exhibits altered nitrogen cycling and reduced arbuscular mycorrhizal fungal associations whereas wild rhizomicrobiome typically maintain more balanced methane metabolism and greater metabolic functional diversity (Chang et al., 2025b).

Precisely, real-time PCR and metagenomic analyses have shown a higher abundance of nitrogen-fixing genes (e.g., *nifD*, *nifH*, *nifK*) in the rhizosphere of microbiomes of wild rice varieties (Chang et al., 2025a), while domesticated rice rhizospheres showed an increased number of genes that are associated with nitrous oxides (N<sub>2</sub>O) production (Chang et al., 2025a), a potent greenhouse gas with approximately 300 times the global warming potential of carbon dioxide. The differences in microbial community structure extend beyond functional gene abundances to encompass distinct

taxonomic compositions. Wild rice rhizospheres are dominated by bacterial families such as *Nitrospiraceae*, *Geobacteraceae*, and *Syntrophaceae* which are frequently associated with beneficial processes including nitrogen fixation and nutrient cycling (Chang et al., 2025a). In contrast, domesticated rice rhizospheres exhibit a markedly different composition characterised by *Bradyrhizobiaceae*, *Syntrophaceae*, *Nitrospiraceae*, and *Anaeromyxobacteraceae* with a significant enrichment of genes involved in denitrification pathways (e.g., *niK*, *nirS*, *norB*, *norC*) that lead to the production of N<sub>2</sub>O (Chang et al., 2025b).

### **1.3.2 Impact of Domestication on Beneficial Microbial Communities**

The loss of microbial diversity during domestication has profound implications for rice plant health and productivity. Comparative studies across multiple crop species, including beet and maize, reveal that wild ancestors possessed significant higher rhizobacterial abundance and diversity compared to modern cultivated varieties (Huang et al., 2022). This reduction in microbial diversity stems from uniform agricultural practises applied to domesticated crops, which contrast sharply with the broader range of environmental stresses that wild plants naturally encounter (Khan, 2023). The consequences of this microbiome simplification are substantial: domesticated rice microbiomes are more sensitive, to pathogen invasion and more easily destabilised than those of wild relatives, which maintain greater relative abundance of beneficial bacterial and fungal taxa (Shi et al., 2018). Evidence from pathogen challenge experiments demonstrates that *Magnaporthe oryza* abundance was significantly lower in wild rice than in domesticated rice when inoculated, indicating that wild microbiome provides superior natural defense against this pathogen (Gutierrez and Grillo, 2022)

The mechanistic basis for these differences lies partially in altered root exudates composition. Root exudates function as key chemical signals that modulates rhizosphere microbiota recruitment and assembly. A study demonstrated that wild rice root exudates positively correlate with frequency and abundance of microbial nitrogen-fixing genes, and when experimentally added to soil, these exudates increase nitrogen-fixing gene abundance and nitrogenase activity (Chang et al., 2025a). Conversely, domesticated rice exudates show no such correlation with nitrogen-fixing genes and even exhibit negative associations with some *nif* genes (Chang et al., 2025a). This bidirectional relationship - where root exudate composition simultaneously reflects and shapes microbiome assembly - indicates that domestication has altered the plant's chemical signalling

capacity, fundamentally changing how microbial populations are recruited and which functions are expressed in the rice rhizosphere.

The reliance on external nitrogen fertilizers during crop intensification has reinforced this trend. As agriculture become increasingly dependent on synthetic nitrogen inputs to boost yields, there was a reduced selective pressure or active selection against the wild type of capability to recruit nitrogen-fixing bacterial communities (Dakshayini et al., 2025). Consequently, domesticated rice may have lost or downregulated genetic features that enables recruitment and maintenance of specific beneficial nitrogen-fixing bacteria. This fertilizer dependency has led to a demonstrable reduction in the abundance and biodiversity of nitrogen-fixing bacteria in domesticated rice rhizospheres (Ramakrishnan et al., 2019), creating a self-reinforced cycle where plants become increasingly dependent on external inputs and less able to exploit endogenous microbial services.

#### **1.4. Genetic Basis of Rice-Microbiome Interactions**

##### **1.4.1 Influence of Rice Genotype on Microbiome Assembly and Function**

The genetic makeup of the rice plant plays a fundamental role in shaping the structure, assembly, and function of its associated microbiome. Genetic makeup can have an impact on the microbiome through microbial selection, root exudates, and functional roles (Singh et al., 2022). According to microbial selection, different rice genotypes can have separate microbial communities, resulting in changes in microbial diversity even when grown in the same soil. This points to a substantial host genetic influence over microbiome makeup (Zhong et al., 2025). The chemical composition of the root exudates, or substances secreted by the roots, is influenced by their genetic makeup. These exudates give nutrients and act as signalling molecules, attracting certain bacteria, and establishing a microbial niche in the root zone. In addition, variation in genes associated to cell wall composition, defence response, or metabolite production might lead to varied root habitats that favour the colonisation of certain microorganisms over others. (Vacheron et al., 2013)

Genome-specific microbiomes have functional implications. A well-adapted microbial community that is specific to its host genotype and delivers benefits such as enhanced nutrient uptake or disease suppression may help the rice variety's overall fitness and productivity. In contrast, an incompatible or less helpful microbiota may lead to inferior performance. Research has demonstrated that some rice types are more effective at attracting specific plant growth promoters

(PGPs) or arbuscular mycorrhiza (AMFs), resulting in differences in nutrient uptake and drought tolerance. Metagenomics analysis of the effect of rice genotypes on microbial assembly revealed that genotypes of the rice plant selectively recruit the microbial community of the rhizosphere, which improves the availability of N, P, and K while reducing greenhouse gas emissions, thus directly or indirectly increasing - yields. These results give a roadmap for developing strains that exploit the benefits of beneficial microorganisms. (Xiong et al., 2021)

#### **1.4.2 Role of Root Exudates in Shaping Rhizosphere Communities**

Root exudates are a complex mixture of low-and high-molecular weight organic compounds released into the soil surrounding roots, forming the rhizosphere. They include primary metabolites such as sugars (e.g. glucose, fructose, sucrose), amino acids (e.g. glutamate, aspartate, alanine) and organic acids (e.g. malate, citrate, fumarate, and oxalate), as well as secondary metabolites such as phenolic acids, flavonoids, alkaloids, phytohormones and various enzymes and proteins. These exudates can account for more than 40% of plant photosynthate released below and represent a major carbon and energy source for rhizosphere microorganisms (Koo et al., 2004; Ma et al., 2022; Salem et al., 2022)

Across plant species, the dominant quantitative components of root exudates are typically organic acids, sugars, and amino acids, with phenolics and other secondary metabolites contributing to smaller but functionally critical fraction. Organic acids such as malic and citric acid are particularly important for nutrients mobilisation, including solubilisation of phosphate and micronutrients, thereby indirectly shaping which microbes can proliferate in the rhizosphere. Secondary metabolites (e.g. phenolic acids, and flavonoids) often function as highly specific signalling molecules, selectively attracting or repelling microbial taxa and modulating microbial gene expression, chemotaxis and biofilm formation (Feng et al., 2021; Koo et al., 2004; Ma et al., 2022; Salem et al., 2022; Schwalm et al., 2024)

The composition and quantity of root exudates are strongly influenced by plant genotypes developmental stage, and environmental conditions (nutrient availability, water status, biotic stress). For instance, contrasting genotypes grown under phosphorus limitation show genotype-dependent shifts in exudation of carbohydrates, amino acids, phenolic and carboxylates, with significant effects of both genotype and P status on exudation rates and on the proportion of each compound in total exuded carbon. These dynamic changes in the exudates are tightly linked to

changes in rhizosphere microbial community structure and function (Kim and Lee, 2020; Liu et al., 2025; Salem et al., 2022; Schwalm et al., 2024; Singh et al., 2022).

#### **1.43 Exudate-Mediated attraction of *Kosakonia sacchari*.**

Chemotaxis towards root exudates is a critical first step in the recruitment and effective colonisation of plant growth promoting rhizobacteria (PGPB). For *Kosakonia sacchari*, transcriptomic and functional studies in medicinal plant system (*Radix pseudostellariae*) have shown that phenolic acids in root exudates act as key chemoattractant. In particular, the phenolic aldehyde vanillin strongly stimulate *K. sacchari* by upregulating genes involved in bacteria chemotaxis, flagellar assembly, glycolysis/gluconeogenesis, the pentose phosphate pathway, fatty acid biosynthesis, and phosphotransferase systems. Vanillin exposure enhances *K. sacchari* motility, biofilm formation and rhizosphere proliferation, thereby promoting more effective colonisation of the root surface and surrounding soil (Feng et al., 2021; Wu et al., 2017)

In the same system, *K. sacchari* can metabolize vanillin into protocatechuic acid which in turn suppresses growth and biofilm formation of beneficial *Bacillus pumilus*, illustrating how bacterial transformation of exudates-derived phenolic can further reshape the rhizosphere community. Although these studies were conducted in the context of replant disease where *K. sacchari* behaves as a pathogen rather than a mutualistic endophytes, the underlying mechanisms - chemotaxis towards specific phenolic exudates, activation of motility and colonisation genes, and utilisation of phenolic as carbon sources are highly relevant to understanding how *K. sacchari* responds to root exudates in other hosts, including rice (Wu et al., 2017).

### **1.5 Genetic and Genomic Approaches for Trait Discovery**

*This section examines the principal genetic and genomic approaches that have been employed to identify loci and alleles underlying traits of agronomic importance in rice, with emphasis on abiotic stress tolerance (e.g., heat, drought, salinity, or sub-optimal nitrogen conditions)*

#### **1.5.1 Quantitative Trait Locus (QTL)**

Quantitative trait locus (QTL) mapping is a population-based, statistical genomic technique that identifies chromosomal segments whose allelic variants significantly explains phenotypic variance of a quantitative character. In rice, the procedure typically merges (i) construction of a high-density

genetic map in a segregating population with (ii) multiple-environmental phenotyping and (iii) interval or composite -interval mapping that calculates logarithms of odds (LOD) scores (Jia et al., 2019; Sabar et al., 2019). Genomic regions exceeding the empirical LOD threshold are declared QTLs (Jia et al., 2019). Subsequent fine mapping, haplotype analysis and gene expression assays turn the anonymous QTL into a candidate gene or even a causal polymorphism ready for marker-assisted breeding (Jiao et al., 2024; Li et al., 2011).

QTL mapping is well suited for examining abiotic stress tolerance, which is a polygenic phenomenon in rice (Sabar et al., 2019; Yi et al., 2023). Researchers have successfully implemented a strategy to control the response to drought (qDTY), salinity (SDS, SKC1), frost (qCTS10<sup>22/23</sup>), heat (qHTSF), and nutrient deficiency, often by contrasting tolerant soils or wild accessions with more sensitive cultivars (Jo et al., 2025; Singh et al., 2018; Vikram et al., 2011; Visakh et al., 2024). At the same time, this approach provides breeders with DNA markers that are strongly associated with beneficial alleles, allowing for immediate adoption through marker-assisted backcrossing (Jiao et al., 2024; Yi et al., 2023).

A recent illustration of QTL-guided discovery for seed-related traits is provided by an IR64 × Hawara Bunar RIL study (Satrio et al., 2025). Using 873 bin-mapped SNPs, the researchers discovered two stable QTLs on chromosomes 2 and 7, which explain 36% of the variance in grain length, width, and thousand-grain weight. Positional cloning and transcript profiling highlighted *OsCW-ZF7*, *FZP* and several seed-storage glutelin genes as the most probable candidates, offering direct targets for improving both yield and nutritional quality in high-yielding backgrounds (Satrio et al., 2025). Another study reported from a 93-11 × *Milyang352* doubled-haploid population screened under 13°C cold-water stress. A major QTL, *qCTS1022/23*, on chromosome 10 (LOD > 8) was shown to co-localise with *Os10g0409400*, a glycosyl-transferase family gene whose tolerant haplotype accelerates seedling recovery and early vigour under low temperature. Markers flanking this QTL are now being introgressed into elite cultivar lines, showing how QTL discovery translates rapidly into climate-resilient cultivar development (Jo et al., 2025).

Once QTLs are identified and validated, marker-assisted selection (MAS) can be used for transfer beneficial alleles from donor parents into elites breeding lines through marker-assisted backcrossing (MABC), enabling early selection and reducing the influence of environmental variation on trait assessment (Shu and Wu, 2016).

### 1.5.2 Genome-Wide Association Study (GWAS)

Genome-wide association studies (GWAS) are a technique used for mapping quantitative trait associated loci, investigating large collection of genotypes, not bi-parental populations, linking single-nucleotide polymorphism (SNP) markers to phenotype. (Zhang et al., 2023). By exploiting historical recombination accumulated in natural or breeding populations, GWAS achieves mapping resolutions that often approach the single gene level, an order of magnitude higher than conventional QTL mapping in biparental families. The technique is now commonplace for analysing polygenic agronomic, quality and stress resistance traits in rice and other crops is usually applied to 1-4 million SNP datasets produced by whole genome resequencing or high-density arrays. Based on phenotype evaluation and genotyping, GWAS is an effective technique to isolate genes related to the target trait. For example, using GWAS, researchers found that *OsSPL13* controls the grain length (Si et al., 2016) and *GSE5* controls grain width (Duan et al., 2017), and the *bsr-d1* allele is associated with Blast resistance (X. Xu et al., 2020). Quantitative trait loci mapping using GWAS discovered genetic areas related with complex traits in several crops, providing possibilities for marker-assisted selection. (Quero et al., 2018).

### 1.5.3 Introgression Lines

Introgression Lines (ILs), also referred to as Chromosomal Segment Substitution Lines (CSSLs) or Backcross Inbred Lines (BILs) or Chromosomal Segments, are a specialised type of biparental genetic population. They are developed through repeated backcrossing and self-pollination to transfer chromosomal segments from a donor parent (often a wild relative or a traditional variety) into the genetic background of a recurrent parent (Zhang et al., 2022). ILs are developed over multiple generations, with 3-6 backcross generations followed by several rounds of self-pollination. This process can be used to transfer desired characteristics from an exotic variety (e.g. wild rice) into an adapted elite cultivar (Zhang et al., 2022). The outcome is a library of “near isogenic swaps” that partitions the entire donor genome into a set of overlapping or non-overlapping ILs, each carrying less than 5% exotic DNA (Samantara et al., 2022). Introgressed segments are extensively tracked using Marker-Assisted Selection (MAS) to speed up the recovery of the recurrent parent genome (Zhang et al., 2022). High-throughput single nucleotide polymorphisms (SNP) genotyping has increased the precision and efficiency of Introgression Lines construction. (Ali et al., 2018).

Because the residual genetic background remains uniform to the population, any phenotypic shift between an IL and recurrent parent (RP) can be attributed to the specific introgressed segments. This unique architecture converts quantitative traits into quasi-Mendelian units rendering them amenable to fine mapping, cloning and functional characterisation. (Zhang et al., 2022). The primary advantage of Introgression Lines in rice development lies in their unique capacity to dissect complex polygene traits, such as drought tolerance or yield, into discrete chromosomal segments, thereby enabling the precise identification and characterization of beneficial quantitative trait loci (QTL) that are otherwise obscured by genetic background noise and epistatic interaction in conventional mapping population (Nyine et al., 2020). This precision allows breeders to directly evaluate the agronomic effects of a specific allele from a donor parent in an otherwise elite genetic background. ILs serve as both a powerful tool for high-resolution genetic mapping of complex traits and as immediate pre-breeding material, where superior lines can be rapidly deployed in breeding programs or even as new varieties, significantly accelerating the development of climate resilience and high-performing rice cultivars (Zhang et al., 2022).

Concrete evidence that ILs can turn exotic varieties into commercial value is provided by the following examples (i)Yield enhancement from *O. rufipogon*: A set of 159 BC<sub>3</sub>ILs (IR64 background × *O. rufipogon*) was phenotyped under low input conditions, and two QTL hotspots on chromosomes 1 and 2 were repeatedly detected. Fine mapping of the chromosome-1 segments delimited a 6.1 kb region harbouring Yld1.1, a gene encoding an AP2 domain transcription factor. When the *O. rufipogon* allele was backcrossed into three elite varieties, it increased grain yield by 10-15% under drought conditions without reducing grain quality (F. Zhang et al., 2021).

(ii)Blast resistance from *O. rufipogon*: Starting from a population of *Swarna* × *O. Rufipogon* BC<sub>2</sub>IL, a study found IL134 that remained blast-free across six hotspots in India. Marker saturation and expression profiling localising the resistance to a 70kb segment on chromosome 12 containing the cloned gene Pi54. The segments were subsequently introgressed into the high-yielding but blast-susceptible variety Samba Mahsuri by two additional marker-assisted backcrossing (MABC) cycles. It generated the released cultivar “*Swarna Pi54*” which retained 97.3 % of the recurrent parent genome while exhibiting durable resistance against 200 *Magnaporthe oryzae* isolates (Basavaraj et al., 2021). These examples underscore that introgression lines not only uncover

hidden beneficial alleles from the wild rice but also deliver them in an elite, marker-defined form ready for immediate cultivar development.

### 1.5.3.1 *Oryza rufipogon* as a Donor for Stress Resilience

*Oryza rufipogon*, the direct ancestor of cultivated rice (*Oryza sativa*) is a valuable reservoir of genetic diversity that has been ended during domestication (Kim et al., 2016). It harbours a wide range of beneficial alleles for abiotic and biotic stress tolerance and yield enhancement traits that may have been lost during domestication and intensive breeding (F. Zhang et al., 2014). The benefits of *O. rufipogon* include drought, salt, and cold tolerance. On drought tolerance, several studies have identified QTLs for drought resistance in *O. rufipogon-derived* ILs (Zhou et al., 2006). The direct use of *O. rufipogon* in agriculture for production is fundamentally impractical due to its undesirable wild characteristics, including severe seed shattering, prolonged seed dormancy, tall plant structure with lodging, low grain yield, and uneven maturity. These traits are opposed to the goal of modern agriculture, which selects non-shattering, high yield, and short-stature and uniform-maturity plants (N. Singh et al., 2020).

With ILs, researchers can use these beneficial alleles without the “linkage drag” of unwanted wild traits. Several successful examples of traits introgressed from *O. rufipogon* into cultivated backgrounds are summarised in Table 1

Table 1 Key Stress Tolerance Traits Introgressed from *O. rufipogon* and Wild relatives

<b><i>Trait</i></b>	<b><i>QTL / Gene</i></b>	<b><i>Chromosome</i></b>	<b><i>Phenotypic Effects</i></b>	<b><i>Reference</i></b>
Drought Tolerance	qDTY1.1	Chr 1	Maintain yield under severe drought stress (15% vs parents)	(X. Xu et al., 2020)
Deep Rooting	<i>DR01</i>	Chr 9	Increases root angle and depth (30% root mass at depth), enhancing drought avoidance	(Singh et al., 2021)
Drought survival	<i>qSDT1.2-2</i>	Chr 12	Improves seedling survival rate under water deficit	(Wang et al., 2017a)

Blast Resistance	<i>Pi54rh</i>	Chr 11	Confers broad spectrum resistance to <i>Magnaporthe oryzae</i>	(X. Xu et al., 2020)
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### 1.5.3.2 Vialone nano: The Recurrent Parent

Vialone Nano is a high-quality Italian japonica rice cultivar known for its superior grain quality and adaptation to a temperate climate. Vialone Nano is a specific Italian variety-within the *Oryza sativa* species (Bosso, 2023), released in 1945. Its key strength lies in its exceptional culinary quality; it has high starch amylopectin content that confers the desired creamy texture and ability to absorb flavours without becoming mushy. This trait is known as “mantecature.” Because of the high quality, the rice has a strong consumer preference and has an established niche market (Sun et al., 2011; D. Xu et al., 2020).

By using Vialone Nano as the recurrent parent in an IL population it is possible to introduce specific stress -tolerance mechanism from *O. rufipogon* while preserving the elite grain quality that deified the commercial value of Vialone nano.

Vialone nano's major limitations are its susceptibility to abiotic stresses. Vialone Nano is sensitive to drought and salinity, limiting its yield under climate stress conditions. It lacks the genetic architecture for water deficit tolerance; this makes it vulnerable to the increased fluctuations in water availability caused by climate change (Trotti et al., 2024). Recent studies have successfully used Vialone Nano as a recurrent parent in IL development, for example, blast resistance ILs were developed by pyramiding multiple R genes (*Piz*, *Pib*, *Pik*, *Pita*) into Vialone Nano using marker-assisted backcrossing (MABC). These ILs retained the grain quality of the recurrent parent while gaining durable blast resistance (Zampieri et al., 2023).

### 1.5.4 Marker Assisted Selection (MAS)

Marker-assisted selection (MAS) is defined as a breeding technique which uses information on the chromosomal location of genes and specific alleles to indirectly select for a trait by selecting molecular markers that are closely related to that trait. This approach allows early selection and reduces the impact of environmental variation on the assessment of the characteristics of the trait (Shu and Wu, 2016).

## **1.6 Climate change and Smart Agriculture for the future**

*This section focuses on recent findings on how climate change is reshaping rice production, emphasising projected yield and quality declines under future climate scenarios. It also investigates smart agriculture and adaptation solutions, such as climate resilience, irrigation innovations, and biofortification, which provide avenues to long-term productivity and nutritional security.*

### **1.6.1 Recent Studies on Climate Change Impacts on Rice**

Recent literature highlights the quantifiable vulnerability of rice production systems to climate change, especially in the context of rising temperature, altered precipitation patterns, and elevated atmospheric carbon dioxide. Climate change poses quantifiable threats to rice production, with projections indicating approximately a 10% decline in yield for every 1°C increase in growing season temperature (Peng et al., 2004). Additionally, altered precipitation patterns are expected to increase the frequency and severity of drought events in around 60% of rice growing regions by 2050 (Calvin et al., 2023).

A comprehensive review by Saud et al. (2022) shows that climate change has already altered rice phenology across China, with a northward shift of the rice planting areas and a shortening of the growth period of both single and double cropping systems. Their findings indicate that although historical yield trends have remained relatively stable due to varietal improvements and agronomic adjustments, future projections under warming scenarios 1.5-2.0°C could reduce national rice yield up to 10.6%, with hotspots such as the Sichuan and Yangtze River basins facing increased heat and drought risk (Saud et al., 2022).

Similarly, a meta-analysis by (Li et al., 2024) synthesising data from more than 100 simulation studies confirms that rising average temperature and precipitation variability are the main climatic drivers of the decline in rice yields. Their quantitative analysis shows that for every 1°C increase in temperature, rice yields fall by 3.85%, with excess rainfall above 25 % of the background level exacerbating the loss of yield. Elevated CO<sub>2</sub> levels (up to 800ppm) can partially offset these negative effects, increasing yield by up to 7.1% per 100-ppm increase; however, this benefit is reduced under severe heat and drought stress.

Beyond yield, climate change also affects grain quality and nutritional value. It was shown (Rezvi et al., 2023) that elevated CO<sub>2</sub> and temperature reduce grain protein as well as key micronutrients such as iron (Fe) and zinc (Zn), along with certain vitamins, raising serious concerns for nutritional security in rice-dependent populations. Together, these findings point to a future in which rice cultivation must simultaneously address two intertwined challenges: maintaining or increasing productivity while preserving grain nutritional quality under increasingly adverse climate conditions. The incidence and severity of diseases such as sheath blight (*Rhizoctonia solani*) are expected to increase due to climate change related higher temperature and humidity. Longer growing periods and warmer nights could accelerate the spread of disease and allow the pathogen to flourish in regions that were previously unsuitable. The combination of climate warming and elevated CO<sub>2</sub> levels creates ideal conditions for sheath blight (Singh et al., 2025).

### **1.62 Water Scarcity**

Rice is one of the most water intensive cereal crops in the world. It accounts for between 34-43% water of total irrigation water uses globally, and it withdraws between 24-30% of fresh water. In many regions, rice irrigation alone represents more than 80% of freshwater consumption. The amount of water required to produce 1kg of unmilled rice averages around 2500 litres with variations ranging between 800 to 5000 litres depending on climate conditions, soil type management practise and cultivation (Surendran et al., 2021a)

In comparison to other major cereals rice has a higher water requirement:

Rice: 2800-3000 litres per kg

Wheat: 1000-1650 litres per kg

Maize: 800-900 litres per kg (Admin, 2023; Ramón, 2022; Surendran et al., 2021a).

Rice thus requires 2-3 times more water than wheat and 3-5 times more than maize for equivalent grain production. This increased water demand is attributed to the rice's flooded paddy cultivation system, which maintains standing water throughout the growing season resulting in high losses through evaporation, seepage and percolation (Ramón, 2022; Surendran et al., 2021a).

It is projected that 15-20 million hectares of irrigated rice may experience water scarcity from 2035 to 2050 and climate change will intensify both drought frequency and competition of freshwater resources across multiple sectors (Richards and Sander, 2014; Surendran et al., 2021a).

### **1.6.3 Adaptation Measures and Smart Agriculture Strategies**

In response to these expected consequences, numerous adaptive methods and smart agricultural technologies have been proposed and, in some cases, effectively deployed. (Li et al., 2024) identified numerous beneficial adaptation strategies, including the use of climate-friendly rice varieties, changing planting dates, and improving irrigation systems. Among these, irrigation optimisation, and variety substitution (replacing traditional variety with stress-tolerant cultivars) demonstrated the greatest promise, improving yields by 11.39% and 2.29%, respectively. The combination of numerous adaptation tactics, including integrated nutrient management, energy-saving approaches, and stress-tolerant cultivars, has resulted in the greatest improvements, with yields increasing by up to 14.59%. Locally appropriate adaptation frameworks and emphasises the importance of smart climate rice technologies that combine productivity, resilience, and mitigation benefits have been advocated by (Saud et al., 2022). These include the production of high-yielding, stress-tolerant varieties using marker-assisted breeding and genome editing, as well as agronomic advances including alternate wetting and drying (AWD) irrigation and water strategies that improve water efficiency and reduce methane emissions.

#### **Alternative Wetting and Drying (AWD): A Water Saving and Climate -Smart Technology**

Alternative wetting and drying (AWD) is a water-management technology designed for irrigated lowlands rice cultivation that significantly reduces water use compared to traditional flooding systems. In AWD rice fields are periodically allowed to dry for several days before re-irrigation. This is done without inducing water stress in the plants. This controlled irrigation scheduling alternatives between flooded and non-flooded conditions throughout the growing season (“Rice farming: saving water through Alternate Wetting Drying (AWD) method | FAO,” n.d.; Richards and Sander, 2014).

AWD delivers multiple environmental, economic, and agronomic benefits. These includes:

1. Water saving: AWD reduces total irrigation water use between 15-30% compared to continuous flooding, this is due to elimination of unproductive water losses through seepage (LaHue et al., 2016; Richards and Sander, 2014; Surendran et al., 2021b)
2. Greenhouse gas mitigation: AWD significantly reduces methane emissions from rice paddies by 48-74% by interrupting the anaerobic soil conditions that favour methanogenic bacteria (LaHue et al., 2016; Richards and Sander, 2014)

## **1.7 Biofortification: Enhancing the Nutritional Quality of Rice**

Improving the nutritional quality of rice represents a critical challenge for global health, particularly in low- and middle-income countries micronutrient deficiencies are prevalent This section explores biofortification strategies, conventional breeding and biotechnological approaches that are aimed at enhancing the iron (Fe) and provitamin A content of rice grains to combat hidden hunger.

### **1.7.1 The Micronutrient Deficiency Challenge**

Iron and zinc deficiencies are among the most widespread nutritional disorders globally, affecting over 2 billion people, particularly in Asia and sub-Saharan Africa where rice consumption is the highest. The most consumed rice, polished white rice, has low amounts of micronutrients due to the removal of the nutrient-rich bran layer during milling. On average polished rice contain only 2-5 µg/g iron and 10-16 µg/g zinc, well below the levels required to meet daily dietary requirement (Gupta et al., 2023, 2023; Kavitha Kumaravel and Srividya, 2025a; Senguttuvel et al., 2023b).

The HarvestPlus biofortification program, part of the CGIAR, has established breeding targets of 13 µg/g iron and 28 µg/g zinc in polished rice, levels designed to provide approximately 30% of the estimated average requirements (EAR) for daily consumption when rice supplies 30% of daily caloric intake. Meeting these targets through biofortification offers a cost effective, scalable and sustainable way to alleviate micronutrient malnutrition without requiring changes in consumer behaviour or diet composition (Gupta et al., 2023; Senguttuvel et al., 2023b; Wairich et al., 2022).

### **1.7.2 Biofortification Approaches: Conventional Breeding and Genetic Engineering**

Conventional Breeding

Natural variation in grain Fe and Zn content is significant and exists within rice germplasm, particularly among traditional landraces, aromatic varieties (e.g. Basmati, Jasmine), pigmented rice types (red rice, brown rice). Coloured rice varieties typically contain 2-3 times higher Fe and Zn concentrations than white rice due to the retention of the nutrient-dense aleurone and bran layers. Systematic screening of rice germplasm collections has identified donor lines with elevated micronutrients concentration and conventional breeding programs have successfully developed biofortified varieties through phenotypic selection and marker assisted breeding (Senguttuvel et al., 2023b).

To date, 37 biofortified rice varieties enriched in Fe, Zn, protein, or provitamin A have been released for commercial cultivation globally (16 from India and 21 from other countries), meeting or approaching HarvestPlus targets. These varieties have been developed through crosses between high-micronutrient donor lines and locally adapted elite cultivars, followed by multi-environment field testing to ensure stable nutrients levels and acceptable agronomic performance (Senguttuvel et al., 2023b).

### **1.73 Transgenic and Biotechnological Approaches**

Genetic engineering strategies have archived greater advances in grain micronutrients content by introducing or overexpressing gene involved in Fe and Zn translocation and storage. Key approaches include:

1. Ferritin overexpression: Ferritin proteins can store iron in plant tissues. Transgenic rice lines expressing soybean (*Phaseolus vulgaris*) or pearl millet (*Pennisetum glaucum*) ferritin genes in the endosperm have achieved 4–7-fold increases in grain Fe content, reaching 30-60 µg/g Fe in polished grains (Gupta et al., 2023).
2. Nicotianamine synthase (NAS) overexpression: *OsNAS2* enhances internal Fe and Zn mobilisation and translocation. Co-expression of ferritin and *OsNAS2* has yielded rice lines with 4.2-fold increases in Fe (up to 56 µg/g) and 3.5-fold increases in Zn (up to 60 µg/g) in polished grains, exceeding HarvestPlus targets (Gupta et al., 2023)
3. Golden rice (Provitamin A biofortification): Golden rice 2, engineered to produce β-carotene (provitamin A) in the endosperm through introduction of phytoene synthase (*psy*) and carotene desaturase (*crtl*) genes, addressing vitamin A deficiency, a leading cause of childhood blindness and immune dysfunction. Golden rice has been approved for cultivation in some

parts of Asia including the Philippines and Bangladesh (Caulfield et al., 2004; Pelletier et al., 1993; Senguttuvel et al., 2023b; Sommer et al., 1983)

### Bioprocess-Based Fortification

Recent innovations include processing techniques such as germination-based fortification, where rice grains are germinated in Fe and Zn enriched solutions (e.g. sodium iron EDTA, zinc chloride). This simple, scalable method increases grain Fe content by 2 folds and Zn content by up to 7 folds while improving bioaccessibility by 2-7fold through reduction phytate, an antinutrient that inhibit mineral absorption. Consumption of 30-150 g per day, of fortified rice can meet 16-70% of daily Fe requirements and nearly 100% of Zn requirements across age groups (Kavitha Kumaravel and Srividya, 2025b)

## 1.8 The Role of Plant Growth Promoting Bacteria in Enhancing Stress Resilience in Rice

### 1.8.1 Diversity of Rice-Associated Diazotrophs

Low oxygen conditions in rice paddy soils favour the growth of a specific group of nitrogen-fixing bacteria (diazotrophs). The community consists of the genera *Azospirillum*, *Azoarcus*, *Herbaspirillum*, and *Enterobacter*. Additionally, three new groups of *Rhizobiaceae* bacteria identified in root zones (rhizosphere) of wild rice (*Oryza rufipogon*) are also part of this selected community (Chen et al., 2014a; Krause et al., 2006; Pelagio-Flores et al., 2025).

*Herbaspirillum* species, particularly *H. seropedicae*, have been extensively studied as diazotrophic endophytes in graminaceous crops. Evidence of plant nitrogen fixation by *Herbaspirillum* ssp strain B501 in wild rice (*Oryza officinalis*) was demonstrated using both acetylene reduction and  $^{15}\text{N}_2$  gas incorporation assay, confirming that these endophytes fix atmospheric nitrogen within rice plant tissues (Elbeltagy et al., 2001). The degree of nitrogen fixation among rice species varies, this suggests host-specific preference or compatibility that may be genetically determined. *Kosakonia sacchari*, isolate originally from sugarcane (*Saccharum officinarum*), has also been identified as a rice endophyte with nitrogen fixing capacity (Chen et al., 2014a). Studies on *Kosakonia* sp strain KO348 demonstrated stable endophyte colonisation in rice roots (between  $10^4$ - $10^5$  CFU/ root tissue) after 5-50 days post inoculation. Type VI Secretion plays a significant role in rhizoplane and endosphere colonisation efficiency (Mosquito et al., 2020). However other field trials with *Kosakonia* sp KO774 inoculation under 50% nitrogen fertilisation showed no significant yield

benefits, this indicates that colonisation capacity does not always translate to measurable growth promotion under field condition and environmental conditions and host genotype can modulate endophyte effectiveness (Mosquito et al., 2020).

Co-inoculation experiments in rice show that the composition of endophytic consortia can strongly influence plant response. Co-inoculation of *Enterobacter cloacea* RCA25 and *Herbaspirillum hettiense* RCA24 at a ratio of 10:1 significantly increased nitrogenase activity (up to 50-60% over RCA25 alone) and enhance chlorophyll, nitrogen content and dry biomass in *Oryza sativa* cv. Baldo under greenhouse conditions, where Vialone Nano showed little benefit due to low colonisation efficiency (Andreozzi et al., 2019). This highlights that selecting compatible endophytes combination tailored to specific host genotype can amplify biological nitrogen fixation and improve physiological performance, highlighting the importance of bioinoculum design rather than relying on single strains.

The same study also revealed strong host specificity in endophyte benefits. While Baldo plants showed higher root colonisation by RCA24 and RCA25 and clear improvements in nitrogen balance index, shoot biomass and clear improvement when inoculated, Vialone Nano exhibited lower root colonisation and no significant gain in growth or nitrogen status relative to uninoculated controls (Andreozzi et al., 2019). These findings reinforce that endophyte performance is strongly genotype-dependent.

## **1.8.2 Mechanisms of Beneficial Plant Microbe Interactions in Rice**

Beneficial microorganisms employ a diverse array of mechanisms to support plant health and productivity under adverse conditions (Ullah et al., 2025). Below are different mechanisms that bacteria employ under abiotic stress.

### **1.8.2.1 Nutrient Acquisition and Solubilisation (N, P, S, Fe, Zn)**

Biological nitrogen fixation (BNF) is the most efficient energy route to deliver  $\text{NH}_4^+$  to roots under low redox conditions typical of water-stressed paddy soils. Beneficial microbes act as living biofertilizers that unlock nutrients otherwise unavailable to stressed plants. Diazotrophs such as *Rhizobium*, *Azotobacter*, and *Azospirillum* fix atmospheric  $\text{N}_2$  into plant-usable ammonium, compensating for nitrogen deficits under drought or salinity. Phosphate-solubilising strains of *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Serratia* excrete organic acids and phosphatases that

release bound phosphorus from Fe/Al oxides or Ca-phosphates (Chaudhary et al., 2022). This increases micronutrient uptake in alkaline or metal-contaminated soils and thereby sustains growth and photosynthetic activity when chemical fertilisers are unavailable or ineffective (Figiel et al., 2025) (Grover et al., 2023)

### **1.8.2.2 Phytohormone Modulation and Stress Signalling**

Microbes in and around the plant root modulate the hormone balance to counteract abiotic stress. They synthesise auxin, indole-3 acetic acid (IAA), that elongates roots and increases absorptive surface under drought or salinity, they secrete cytokinins and gibberellins that delay leaf senescence and sustain chlorophyll under heat and water deficit (Egamberdieva et al., 2017). They produce abscisic acid (ABA) to heighten stomata sensitivity and curtail transpirational loss and release ACC deaminase, an enzyme that cleaves the ethylene precursor ACC into  $\alpha$ -ketobutyrate and ammonia, keeping ethylene concentration below inhibitory levels for root growth, especially under salinity, waterlogging, and heavy metal stress (Kudoyarova et al., 2019).

One of the most characterised bacterial mechanisms for stress mitigation is the production of ACC deaminase (1-aminocyclopropane-1-carboxylate deaminase), an enzyme that cleaves the ethylene precursor ACC into  $\alpha$ -ketobutyrate and ammonia. By reducing ACC accumulation in plant tissues, ACC deaminase-producing bacteria lower ethylene concentrations below levels that would otherwise inhibit root growth, particularly under salt stress, waterlogging, heavy metal toxicity, and drought stress (Ferreira et al., 2025; Kudoyarova et al., 2019). Studies across multiple crop including rice, maize and wheat have demonstrated that inoculation with ACC deaminase - producing strains e.g. *Bacillus*, *Pseudomonas*, *Achromobacter*, *Enterobacter*) under polyethylene glycol (PEG)-induced osmotic stress or field drought conditions significantly improves seedling vigour, root development, biomass accumulation, and antioxidant enzyme activity, effectively alleviating stress-induced growth inhibition ((Ferreira et al., 2025)

### **1.8.2.3 Induced System Resistance (ISR)**

Water-stressed plants are more susceptible to pathogens; for example, water stress predisposes rice to blast (*Magnaporthe oryzae*) by suppressing basal immunity (Cooper and Ton, 2022; Devi et al., 2023). Plant Growth Promoting Bacteria (PGPB) trigger Induced System Resistance, a mechanism which primes the host plant's immune system, leading to a more rapid and stronger defence response against a broad spectrum of pathogens.

In rice, ISR has been demonstrated with multiple PGPB genera. Inoculation with *Pseudomonas* sp strains significantly increases the activity of defense-related enzymes, including peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia-lyase (PAL), and superoxide dismutase (SOD) upon challenge with the sheath blight pathogen *Rhizoctonia solani* (Reshma et al., 2018; Salwan et al., 2023). These enzymes catalyse the oxidation of phenolic compounds into antimicrobial quinones, generate reactive oxygen species (ROS) for pathogen killing, and strengthen cell-wall lignification, thereby restricting pathogen colonisation and spread. Similarly arbuscular mycorrhizal fungi (*Glomus intraradices*) colonising rice roots constitutively elevate POD and PPO activities, priming defense pathways and reducing blast disease severity (Campos-Soriano et al., 2012).

PGPB-induced ISR also extends to abiotic stress tolerance. *Bacillus velezensis* strain *GHI-13* inoculation in rice drought stress significantly enhances drought tolerance by activating the expression of ROS scavenging genes (*OsAPX*, *OsCAT*, *OsSOD*) and suppressing ROS accumulation, thereby reducing oxidative damage to membranes, proteins and DNA (Park et al., 2024). This activation is mediated through jasmonic acid (JA) signalling, as *B. velezensis* upregulate JA biosynthetic genes (*OsLOX*, *OsAOS*), which in turn induce antioxidant gene expression. Under high salinity stress, halotolerant *Bacillus* strains inoculated into rice seedlings significantly upregulate salt stress responsive gene by 4-5 folds, this improves ion homeostasis, osmotic adjustment and membrane stability, ultimately enhancing salinity tolerance (Ali et al., 2022)

Table 2 Key microbial genera and their roles in enhancing abiotic stress tolerance in rice

<b>Microbial Genus / Species</b>	<b>Abiotic Stress Targeted</b>	<b>Mechanism of Action (Direct vs. Indirect)</b>	<b>Key Reference(s)</b>
<i>Bacillus amyloliquefaciens</i>	Salinity, drought	Direct: ↑ IAA, ABA, siderophores, ROS-scavenging enzymes (CAT, SOD, APX)	(Li et al., 2023)
<i>Bacillus pumilus</i>	Salinity, heavy metals (Cd, Pb)	Direct: ACC-deaminase ↓ ethylene; ↑ antioxidant enzymes; siderophore-mediated metal chelation	(Tang et al., 2024)
<i>Pseudomonas fluorescens</i>	Salinity, drought, heavy metals	Direct: ACC-deaminase, IAA, osmolyte induction; Indirect: ISR & ROS detoxification	(Gupta et al., 2022)
<i>Pseudomonas pseudoalcaligenes</i>	Salinity	Direct: ↑ glycine-betaine-like osmolytes; ↓ Na <sup>+</sup> uptake	(Zhu et al., 2022)
<i>Trichoderma harzianum</i>	Salinity, drought	Direct: ↑ aquaporin, dehydrin and antioxidant gene expression; ↑ phenolics & lignin	(Pandey et al., 2016)
<i>Azospirillum brasilense</i>	Salinity, drought	Direct: nitrogen fixation, IAA, polyamine accumulation; ↑ stress-responsive gene expression	(Giri et al., 2025)
<i>Enterobacter ludwigii</i>	Salinity	Direct: ↑ stress-linked genes, ↓ ethylene & ROS	(Wei et al., 2022)
<i>Piriformospora indica</i> (fungal endophyte)	Salinity	Direct: modulates salt-responsive genes; ↑ antioxidant capacity	(Raeisi Vanani et al., 2024)
<i>Glomus intraradices</i> (AMF)	Heavy metal (Cd, As), flooding	Direct: ↓ Cd/As uptake; ↑ micronutrient (Zn, Fe) acquisition; Indirect: improved soil structure	(Xu et al., 2024)

## 1.9 *Kosakonia sacchari*

### 1.9.1 A Model Endophyte for Enhancing Rice Under Abiotic Stress.

*Kosakonia sacchari* is a Gram negative, motile rod shaped and non-spore forming bacteria. It grows aerobically but can fix  $N_2$  to  $NH_3$  at low oxygen concentration. It belongs to the *Enterobacteriaceae* family. Its optimal growth conditions are around 30°C and at pH 7 (Chen et al., 2014b). Some studies have shown that *K. sacchari* can tolerate up to 37 °C (Elhafeez E et al., 2018). Like other bacteria in the *Enterobacter* genera, *Kosakonia sacchari* can utilise a wide range of sugars and amino acids such as D-fructose, D-mannitol, D-mannose, maltose, D-galactose, glycerol and L-alanine (Chen et al., 2014b). *Kosakonia sacchari* was first isolated from sugarcane (*Saccharum officinarum*). After 24 hours on nutrient agar colonies of *Kosakonia sacchari* typically appear light yellow, circular with entire margins, smooth and have convex elevation, (Zhu et al., 2013). Some strains of *Kosakonia sacchari* exhibit notable resilience to diverse abiotic stresses, this positions *Kosakonia sacchari* as a promising candidate for application in agriculture under adverse environmental conditions (Shahid et al., 2021).

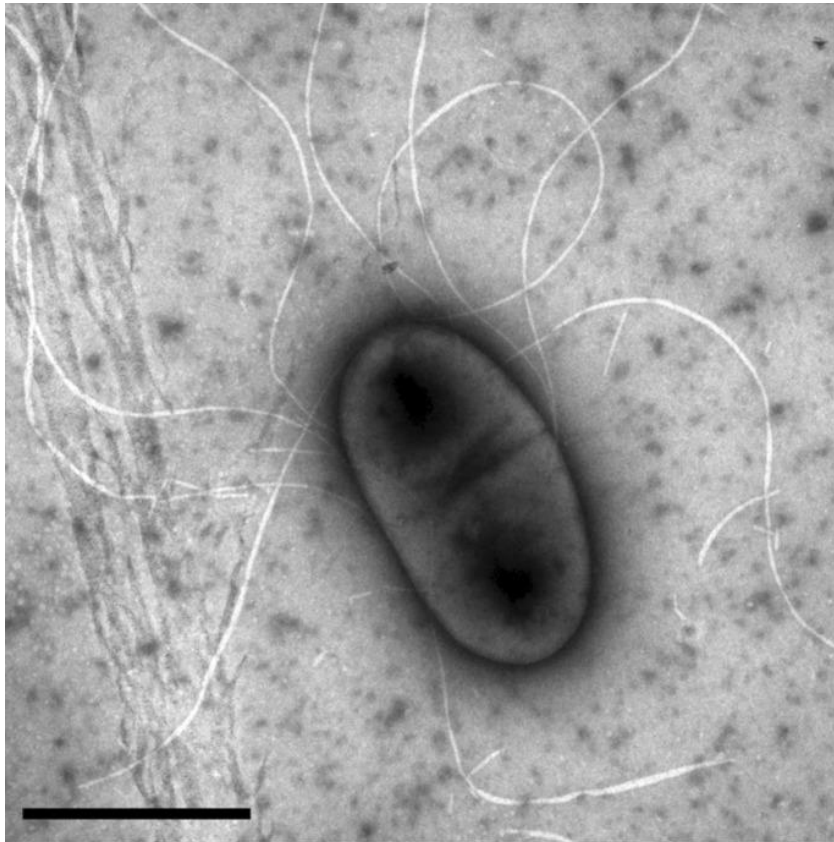


Figure 3: Transmission electron microscope showing a negatively stained cell of the *Kosakonia sacchari* type strain SP1T. (Chen et al., 2014b)

## Taxonomy and classification

The diagram below summarises the taxonomy of *Kosakonia sacchari*.

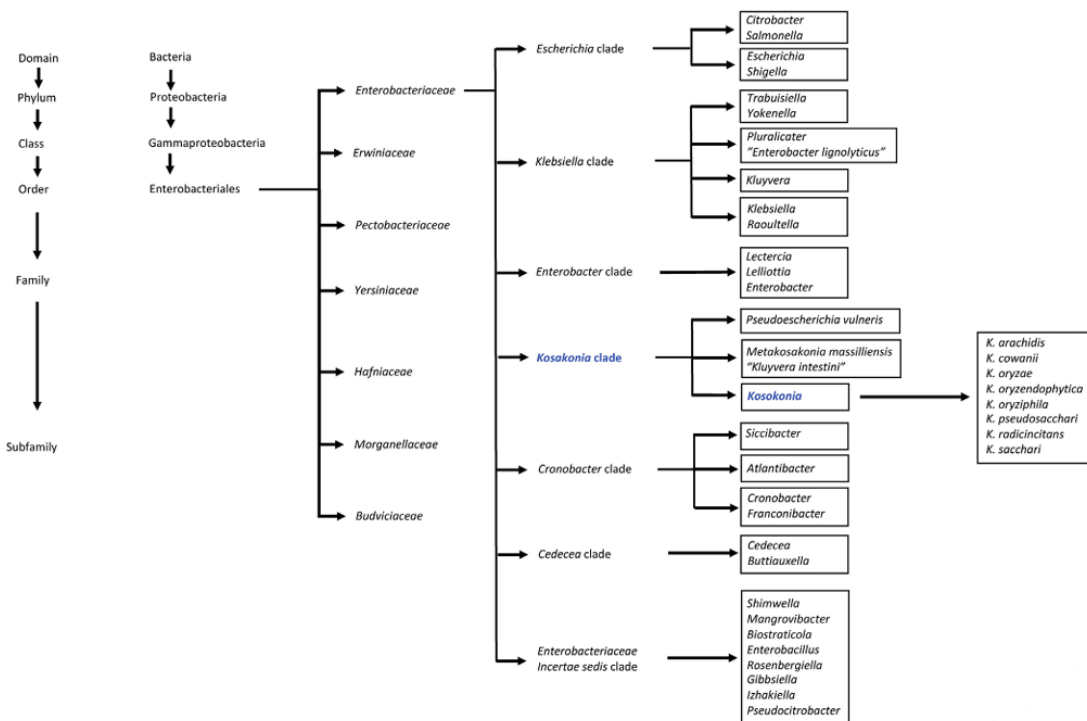


Figure 4 The taxonomic hierarchy of *Kosakonia* genus (“*Kosakonia*,” 2020)

### 1.9.2 Evidence for *Kosakonia sacchari* and related species from recent studies

*Kosakonia sacchari* acts mainly as plant associated bacterium with PGP activity (Chen et al., 2014b). It functions as diazotroph, it possesses *nif* gene cluster, which allows it to convert atmospheric nitrogen into a form usable by the plant host (Li et al., 2017). Recent work positions *Kosakonia sacchari* as a promising epiphytic and diazotrophic model for mitigating abiotic stress in rice, especially under water limited conditions. The bacterium colonises roots and expresses nitrogen fixation, indole -3- acetic production and ACC-deaminase activity, collecting enhancing root architecture and water uptake efficiency (Romano et al., 2020).

A study found that when metabolically active *Kosakonia radicincitans* produces key PGP compounds (IAA, siderophores, ACC deaminase and ammonia), with their production increasing as NaCl concentration rises (Shahid et al., 2022). Another strain in the *Kosakonia* genus, *Kosakonia*

*radicincitans* KR17 was found to alleviate salinity stress with *Raphanus sativus* by improving germination, dry biomass and leaf pigments under salt stress, and significantly increased plant mineral content (Na, K, Ca, Mg, Zn, Fe, Cu, P, N) as well proteins, carbohydrates, root pigment, amino acids, lipids and root alkaloids (Shahid et al., 2022). During a study on *Vigna radiata* colonisation by the halotolerant bacterium *Kosakonia sacchari*, strain MSK1 was found to be metabolically active even at 1 M NaCl. 16S rRNA profiling confirmed its identity and its synthesis of indole-3-acetic acid, ACC deaminase siderophores, ammonia and hydrogen cyanide all rose up in step with increasing salinity (Shahid et al., 2021). The endophytic competence of *Kosakonia sacchari* was verified in rice experiment, a T6SS deficient *Kosakonia sacchari* mutant 70% of wild type endosphere colonisation (Mosquito et al., 2020), proving the secretion system is indispensable for intracellular establishment. Despite *Kosakonia sacchari*'s promise as a stress-mitigating endophyte (Romano et al., 2020), no study has tested its colonisation efficacy across introgression lines or its transcriptional impact on host drought responses. This study addresses this by integrating colonisation patterns, gene expression using RNA seq and phenotyping under drought.

## 2.0 AIM OF THE RESEARCH

The experimental work presented in this thesis was conducted at the *Genetics Laboratory* of the Università del Piemonte Orientale, Vercelli, Italy.

### Aim of the study

The main aim of the study is to map *Kosakonia sacchari* colonisation patterns in Vialone Nano × *O. rufipogon* introgression lines (ILs) across a panel of 41 introgression lines (ILs) and 2 parental lines using CFU counts and RNA sequencing, and test how inoculation affects drought tolerance.

### 2.1 Research Gap and Justification

Domestication has eroded the capacity of elite rice cultivar to recruits nitrogen fixing bacteria and stress mitigating microbes (Chang et al., 2025a; Lei et al., 2025). Wild *Oryza rufipogon* by contrast, harbour root assisted microbiota with superior nitrogen fixing potential and exudate chemistries that favour beneficial colonisation. To date, studies on root colonization in rice varieties e.g., *Oryza rufipogon*, Vialone Nano (a specific variety within *Oryza Sativa*) and endophytic bacteria have primarily focused on limited introgression lines derived from *Oryza rufipogon* × *Oryza sativa* crosses e.g., (Wang et al., 2017a), overlooking both (i) broader panels of introgression lines (ILs) such as those bred from Vialone Nano × *O. rufipogon*, and (ii) key bacteria like *Kosakonia sacchari*, despite its demonstrated role in nitrogen fixation under saline stress (Romano et al., 2020).

Consequently, current literature lacks a comprehensive understanding of:

- i. Quantified colonisation dynamics across a panel of introgression lines (ILs) derived from Vialone Nano × *Oryza rufipogon*.
- ii. Transcriptional responses associated with differential colonisation, particularly genes and pathways differentially regulated during symbiosis in high versus low colonising lines
- iii. Rice genetic loci underlying colonisation variation, correlating root colonisation (CFU counts) with genetic variation identified through whole-genome sequencing; or
- iv. Functional consequences of colonisation under abiotic stress, specifically whether *K. sacchari* inoculation enhances water stress tolerance in ILs.

Given that *K. sacchari* possesses multiple plant growth promoting traits (nitrogen fixation, IAA, (reactive oxygen species) ROS scavenging enzymes (Shahid et al., 2022), we hypothesise that ILs exhibiting high colonisation will display a distinct transcriptional signature under drought, enriching for genes involved in stress, hormonal signalling and root development. Current breeding programs therefore lack the knowledge required to select for improved microbe-host compatibility as route to drought resilience. To address this, an integrated experimental workflow was designed that include, quantitative microbiology, controlled phenotyping (Shoot Length, Relative Water Content) and RNA sequencing.

## **2.2 Overall Project Biological Question and Thesis specific contributions**

This thesis is part of a larger collaborative research project investigating *Kosakonia sacchari* colonisation and its role in stress tolerance in rice introgression lines. The overall projects aim to address the following biological questions:

1. What is the variation in *Kosakonia sacchari* root colonisation (CFU/g root) across a panel of nearly 400 introgression lines (ILs) from Vialone Nano × *Oryza rufipogon*, and which ILs show statistically significant high/low colonisation phenotypes?
2. Which genes and biological pathways are differentially expressed (RNA-seq) in high-colonising ILs versus low-colonising ILs during *K. sacchari* symbiosis?
3. Does *K. sacchari* inoculation enhance water-stress tolerance in selected ILs grown in RCA25, and how does it affect growth/yield parameters (height, flowering time, chlorophyll, flavonoids, tillering, yield)?
4. Do genetic variants identified through whole-genome resequencing (NGS) of high vs. low-colonising ILs correlate with observed CFU counts, and can these loci be linked to known symbiosis-related pathways?

### **2.2.1 Thesis Specific Contribution and Scope**

Due to the timeline of this thesis and extended time required for RNA sequencing and whole genome sequencing, not all the biological questions could be addressed within the scope of this works. The specific contributions of this thesis are :

1. Colonisation patterns of *K. sacchari* RCA25 were mapped across a panel of 41 introgression lines and 2 parental lines using CFU quantification, identifying significant variation in colonisation affinity across IL panel
2. A controlled drought stress experiment conducted using four selected genotypes (IL189, IL273, Vialone nano, and *O.rufipogon* ), measuring relative water content and shoot length as growth parameters under 50% and 100% field capacity conditions
3. RNA was successfully extracted from roots tissues of the four genotypes stress tested and RNA integrity was assessed using agarose electrophoresis and Agilent 2100 Bioanalyzer. Batch 2 samples confirmed RNA integrity with the majority achieving  $RIN \geq 7$ . Samples meeting quality standards were prepared and submitted for sequencing. Sequencing results have not been received

Biological question 4 has not been addressed within the scope of this thesis. This work is planned as part of the future component of the research project

#### Hypotheses

H<sub>1</sub>: Introgression Lines will exhibit differential colonisation by *K. sacchari* compared to the recipient parent Vialone nano, reflecting the influence *O. rufipogon* alleles.

H<sub>2</sub>: The wild variety *O. rufipogon* will support higher bacterial colonisation than the elite cultivar Vialone nano, *reflecting* preserved microbiome -interacting traits lost during domestication.

H<sub>3</sub>: *K. sacchari* inoculation will enhance water stress response and will influence growth parameters.

H<sub>4</sub>: Host gene expression will differ between genotype in response to bacterial colonisation.

### 3.0 MATERIALS AND METHODS

Introgression lines provide advantages when studying plant-endophyte (*Kosakonia sacchari*) relationships. They act as high genetic resolution of colonisation; by comparing the colonisation density (CFU/g) of an IL against the recurrent parent (Vialone nano), researchers can pinpoint quantitative trait loci (QTLs) that control bacterial recruitment (Sena, 2025). They assist in uncovering “lost” microbiome alleles; domestication has potentially eroded traits required for effective symbiosis. ILs allow for the systematic re-evaluation of wild alleles from *O. rufipogon* that may restore the plant’s ability to recruit beneficial diazotrophs or stimulate bacterial activities through specific root exudates (Chang et al., 2025a)

#### 3.1 Experimental Design

##### 3.1.1 Treated Groups and Replication

The experiment followed a completely randomised design in the second experimental phase.

##### Part 1-Colonisation Mapping Across IL Panels

- Genotypes: 41 introgression lines and 2 parental lines screened
- Treatments: Inoculation with *K. sacchari* RCA25 ( $10^7$  CFU/mL)
- Replication: 5 biological replicates per line
- Sampling: 7 days post-inoculation
- Total experimental units: >200 plants

##### Part 2 -Drought Stress Test on selected ILs and Parental Lines

- Genotypes: IL189, IL273, Vialone nano, *Oryza rufipogon*
- Treatments: Inoculated vs non-inoculated (control)
- Replication: 5 biological replicates per line
- Water regime: 50 % and 100% field capacity (FC)
- Sampling for Homogenization: 7 days post inoculation
- Sampling for Relative Water Content, plant height, and roots for gene expression analysis: 14 days post inoculation

Total experimental units: 4 genotypes × 2 inoculation treatment × 2 water regimes × 2 replicates

#### Section 1: Colonisation Patterns of *Kosakonia Sacchari* in Introgression Lines (IL)

## **3.2 Plant Material and Growth Conditions**

### **3.2.1 Introgression Lines and Parental Lines**

A population of introgression lines (ILs) derived from backcrossing between *Oryza rufipogon* (wild donor) and Vialone Nano(VN, *Oryza sativa ssp japonica* recurrent parent) was developed through the repeated backcrossing and selfing (BC<sub>3</sub>SF<sub>4</sub>) generation. For the present study, a panel of 41 introgression lines and 2 parental genotypes (Vialone Nano and *Oryza rufipogon*) was screened.

### **3.2.2 Seed Sterilisation and Germination**

Rice seeds were dehusked and subjected to sequential sterilisation steps:

1. Immersed in 70% (v/v) ethanol for 5 minutes with gentle shaking.
2. Rinsed twice with 35–40 mL sterile water (12 inversions per rinse)
3. Treated with sterile bleach containing Tween (1000ppm) for 25 minutes under shaking (Multi Bio 3D mini shaker) conditions.
4. Rinsed seven times with 35 mL sterile water.
5. Allowed to rest for 30 minutes in the final sterile water.

Following sterilisation, seeds were transferred to 0.8% (w/v) agar/water plates under sterile conditions using sterile tweezers and incubated at 22°C in complete darkness for 3-5 days (Panasonic Cooled incubator MIR -154 PE) until germination. Seedlings were considered ready for inoculation when a root emergence reached 0.5-1.5cm.

## **3.3 Bacterial Strain and Inoculum Preparation**

### **3.3.1 *Kosakonia sacchari* strain Selection**

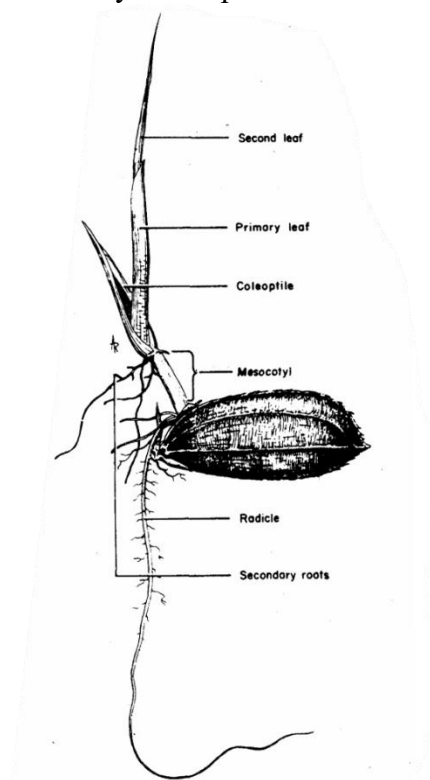
*Kosakonia sacchari* strain RCA25 was selected for this study based on its previously demonstrated plants growth-promoting traits, including nitrogen fixation, phosphate solubilization, IAA (indole-3-acetic acid) production, and ACC deaminase activity (Andreozzi et al., 2019; Romano et al., 2020).

### **3.3.2 Culture Conditions**

Stock cultures of *K sacchari* RCA25 was kept at -80°C.

### Pre-inoculum Preparation

Fresh stock cultures were thawed and inoculated into 3 mL of liquid Lysogeny Broth (LB) medium supplemented with 1000ppm vancomycin in Falcon tubes. Culture was incubated horizontally in a shaking incubator (Multi Bio 3D mini shaker) at 70 rpm for 48 hours at 30 °C. Following initial growth, a 1:1000 dilution was prepared by transferring 1 µL of pre-inoculum into 15 mL of fresh LB medium with 15 µL vancomycin in 50 mL Falcon tubes. These were shaken using mini shaker horizontally at 70 rpm for 24 hours at 30°C.



*Figure 5: Seeding stage at which uniform germination was achieved prior to bacterial inoculation. (Chang and Bardenas, 1965)*

### 3.3.3 Inoculation Standardisation

Prior to inoculation, bacterial cultures were quantified using a Shimadzu UV spectrophotometer UV-1800 to measure optical density at 600nm ( $OD_{600}$ ). The culture was diluted or allowed to grow to achieve an  $OD_{600}$  of 0.8-1.2.

For standardisation to a final concentration of  $10^7$  CFU/mL:

1. Bacterial culture volume required to reach the final cell concentration ( $OD_{600} = 0.8-1.2$ ) was centrifuged (Thermo Scientific Heraeus Fresco 21 Centrifuge) at 8000rpm, 5 minutes, 22 °C
2. The bacterial pellet was resuspended in the same volume of sterile 0.1 M  $MgSO_4$  solution.

3. Serial dilutions were prepared in 0.1 M MgSO<sub>4</sub> to achieve the target inoculum density (10<sup>7</sup> CFU/mL in a final volume of 10 mL)

The dilution equation used was:

$$C_1 \times V_1 = C_2 \times V_2$$

Where:

C1: Initial concentration of the bacterial stock

V1: Volume of bacterial stock needed

C2: Target concentration (10<sup>7</sup> CFU/mL)

V2: Final total volume (10 mL)

The final standardisation inoculum was used for all seedling inoculations to ensure uniform initial bacterial load across the treatment groups.

### **3.4 Inoculation Method**

#### **3.4.2 Seedling inoculation**

Germinated seedlings were carefully removed from agar plates and immersed either:-

Inoculated treatment: Standardised bacterial suspension (10<sup>7</sup> CFU/mL *K. sacchari* RCA25 in 0.1 M MgSO<sub>4</sub>, 10mL total volume)

Control treatment: Sterile 0.1M MgSO<sub>4</sub> solution (10 mL, identical osmolarity and pH)

Both groups were subjected to identical procedures.

1. Seedling immersion for 4 hours at room temperature 23°C
2. Gentle shaking at 140 rpm (Multi Bio 3D mini shaker) in darkness
3. Following immersion, seedlings were transplanted into sterile soil (sand: perlite, 1:1) in 500mL pots (6 plants per pot)
4. Pots were irrigated with 40mL sterile distilled water, covered with plastic wrap to maintain humidity, and transferred to growth chambers (as described in section 3.5)

Rationale for root dip inoculation This method was selected because it (i) ensures uniform initial bacterial contacts with root surface, (ii) effectively establishes endophytic colonisation in rice systems (Andreozzi et al., 2019; Defez et al., 2017a; Flores-Castañón et al., 2025) (iii) mimics natural root-bacterial encounters in field conditions.

### **3.5 Growth Chamber Conditions**

Following inoculation, plants were transferred to and maintained in the growth chamber under the following conditions:

**Light:** 24°C, 16 hours light and 21°C, 8h dark

**Relative Humidity:** 72%

After 72-96 hours, plants were irrigated, and sterile water was sprayed along pot edges.

### **3.6 Quantification of Bacterial Colonisation (CFU Analysis)**

#### **3.6.1 Root Harvesting and Surface Sterilisation**

After 7 days, roots were carefully removed from the pots and surface sterilized to eliminate contaminant bacteria while preserving endophytic population. Surface sterilization was performed via sequential immersion.

- First immersion: 20 mL bleach solution for 1 minutes with gentle agitation
- First rinse: Two 1minute washes in 20mL sterile distilled water.
- Ethanol: immersion 20 mL of 70% (v/v) ethanol for 1 minute with gentle agitation
- Final rinses: Seven 1-minute washes in 20 mL sterile distilled water

During the 7<sup>th</sup> rinse samples were incubated in darkness for 20 minutes to ensure complete sterilant removal.

#### **3.6.2 Root Homogenisation**

Surface-sterilised roots were blotted on sterile filter paper to remove excess moisture and immediately weighed on an analytical balance (Sartorius Lab Instruments GmbH, QUINTIX125D) (to  $\pm 0.01$ g). Roots were then transferred to sterile mortars and ground with a pestle. Homogenisation was performed in the presence of 10 mL of sterile 0.1 M MgSO<sub>4</sub> solution.

The resulting homogenate was transferred to sterile 10mL Falcon tubes. Samples were then shaken in darkness at room temperature 23°C and 140 rpm for 1.5 hours to facilitate bacterial release from root tissue.

### **3.6.3 Serial Dilution and Plating**

Homogenates were serially diluted in sterile 0.1M MgSO<sub>4</sub> according to the following scheme.

1. First Dilution: a 1:2 dilution (1 mL homogenate + 1 mL MgSO<sub>4</sub>)
2. Second dilution: a 1:20 dilutions (150 µL of previous dilution + 1350 µL MgSO<sub>4</sub>)
3. Third dilution: a 1:200 dilutions (150 µL of previous dilution + 1350 µL MgSO<sub>4</sub>,

Aliquots (50 µL) of the serial dilutions were plated onto LB agar plates supplemented with vancomycin (VANC-20) using sterile spreaders. Three replicates were plated per biological sample.

### **3.6.4 Incubation and Colony Count**

Plates were incubated in the dark at 30°C for 24 hours. Following the incubation, the sterility of the control plates (containing only MgSO<sub>4</sub>, no bacterial suspension) was verified. Colonies were counted in plates containing between 30-300 colonies (countable range) using manual counting.

### **3.6.5 CFU Calculation**

Colonisation levels were calculated as CFU per gram of fresh root tissue using the formula:

$CFU/g = [Colony\ count \times (200) \times DF] / \text{root weight (g)}$ , where:

- Colony counts = number of colonies on the plate
- 10mL = total volume of homogenate
- 0.05 mL = volume plated
- $DF = 2/20/200 = \text{final dilution factor (1:200)}$

- Root weight= fresh weight (g) of surface sterilised root tissue

The protocol for Colonisation Mapping across the IL panel can be summarised with the Figure 6 below.

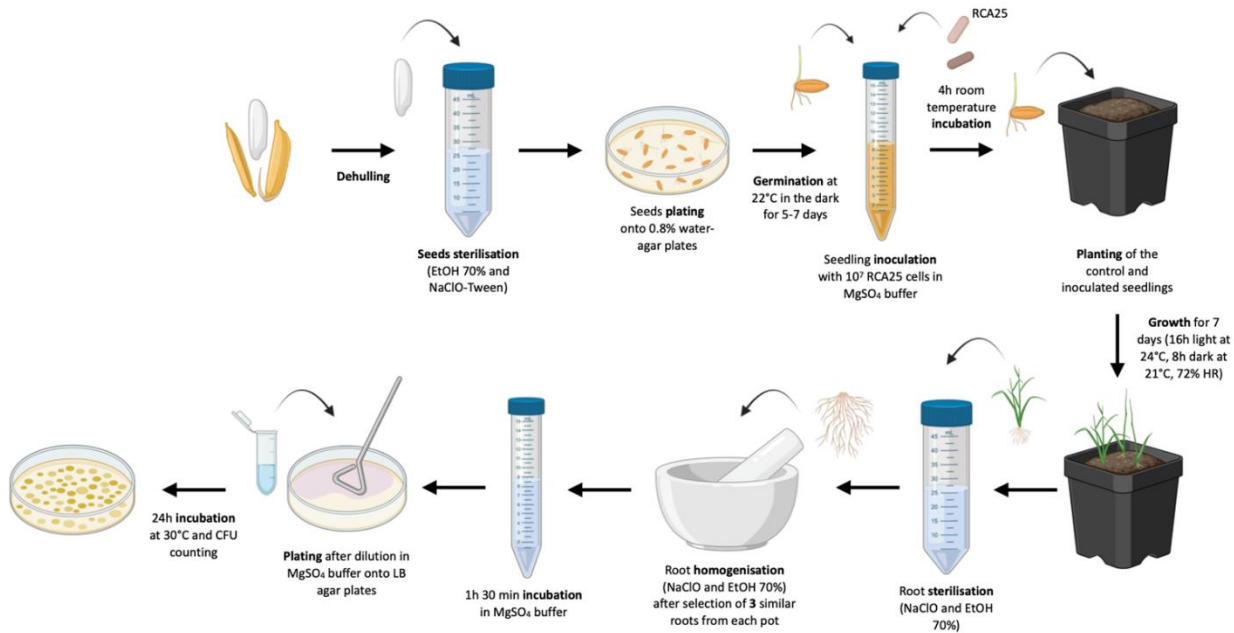


Figure 6: Protocol for seedlings inoculation (unpublished article).

## Section 2: Stress Test of IL273, IL189, Vialone Nano and *Oryza rufipogon*

The drought stress test was conducted following the same experimental workflow as described in Part 1. The four genotypes which were evaluated are IL189, IL273, Vialone Nano and *Oryza rufipogon* 602-131-2. The water stress was applied for 7 days after 1 week from seedling inoculation. After 14 days post inoculation (after 7 days of water stress application), shoot length and root samples were collected and immediately immersed in liquid nitrogen and stored at -80°C. Fresh weight of three leaves was measured the same day. After 7 days turgid weight was measured, then leaves were incubated for 7 days at 50°C and dry weight measured. Final leaf data of were used to calculate the Relative Water Content (RWC) with the following formula:

$$RWC(\%) = \frac{FW - DW}{TW - DW} \times 100$$

Equation 1: Relative Water Content (RWC)

Parameters

FW (Fresh Weight): Mass of leaf immediately after harvesting

TW (Turgid Weight): Mass of the leaf after re-saturation in distilled water for 1 week

DW (Dry Weight): Mass of leaf after drying in an oven (at 50°C) for one week

Schematic representation of the water stress workflow is shown below in Figure 7.

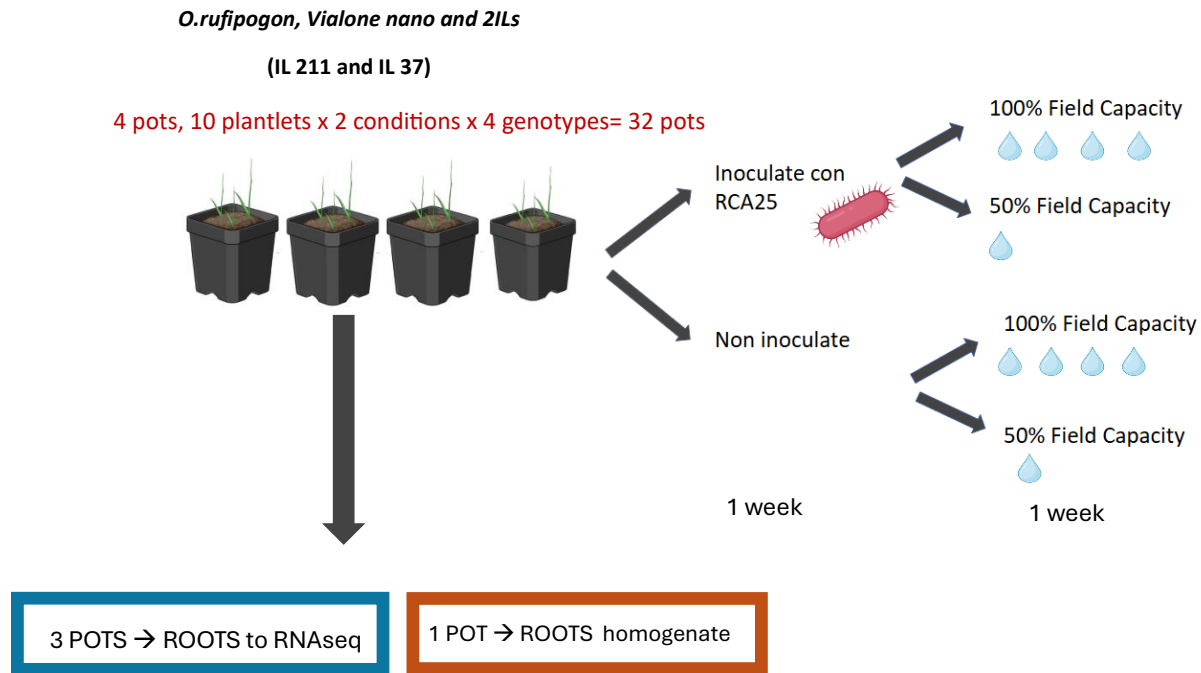


Figure 7: Schematic representation of the water stress test (unpublished article).

### Section 3: DNA and RNA extraction, Gene expression Analysis

#### 3.7 Molecular Analysis

##### 3.7.1 DNA Extraction from Leaves (For Genotyping)

Rationale. DNA was extracted from leaf tissue for genotyping and for future QTL mapping from this study.

##### 3.7.1.1 Sample homogenisation and Lysis

DNA was extracted from rice leaves using NucleoMag Plant kit (Macherey-Nagel) according to the manufacturer's protocol with the following modifications:

1. 50 mg of fresh leaf tissue was homogenised in a microtube.
2. 500  $\mu$ L of Buffer MC1 was added.

3. Tubes were sealed with cap strips, vortexed vigorously for 30 seconds and centrifuged at  $1,500 \times g$  for 30 seconds.
4. Samples were incubated at  $56^{\circ}\text{C}$  for 30 minutes.
5. For RNA-rich tissues,  $10 \mu\text{L}$  of RNase was added to the lysis mixture before incubation.

### **3.7.1.2 Lysate Clarification**

Following lysis, samples were centrifuged at  $8000 \times g$  for 20 minutes at room temperature. Cap strips were removed, and  $400 \mu\text{L}$  of cleared lysate (equilibrated to room temperature) was transferred to a Square-well Block, avoiding contact with the well rims.

### **3.7.1.3 Magnetic Beads Binding**

A suspension of  $30 \mu\text{L}$  NucleoMag C-Beads and  $400 \mu\text{L}$  Buffer MC2 was added to each lysate-containing well. Beads were mixed by pipetting 6 times and shaken for 5 minutes at room temperature. The Square-well Block was placed on a magnetic separator for  $\geq 2$  minutes to immobilise beads.

### **3.7.1.4 Sequential Washes**

Three wash steps were performed sequentially:

**Buffer MC3:**  $600 \mu\text{L}$  was added, resuspended by shaking (5 minutes) or pipetting (15 times), separated magnetically ( $\geq 2$  minutes), and supernatant discarded.

**Buffer MC4:** Identical resuspension and separation steps as MC3.

**80% Ethanol:** Identical resuspension and separation, with ethanol removal under magnetic immobilisation.

### **3.7.1.5 Residual Ethanol Removal**

While immobilised on the magnetic separator,  $600 \mu\text{L}$  Buffer MC5 was gently added to each well without resuspending the beads. After 45–60 seconds, the supernatant was aspirated to eliminate residual ethanol, omitting a drying step.

### **3.7.1.6 DNA Elution and Quality Control**

Beads were resuspended in  $50\text{--}200 \mu\text{L}$  pre-warmed Buffer MC6 ( $55^{\circ}\text{C}$ ) and incubated at  $56^{\circ}\text{C}$  for 5–10 minutes with shaking or pipetting. Post-incubation, beads were magnetically separated ( $\geq 2$

minutes), and supernatant containing purified genomic DNA was transferred to an elution plate. Elution efficiency was enhanced by 15–20% using pre-warmed buffer or extended 10-minute incubation at 55°C.

Quality Control: DNA concentration was assessed via the DNA Fluorimeter (Qubit - Thermo Fisher).

DNA samples were diluted to 50 ng/μL for downstream genotyping applications. DNA is used as the “reference genome” needed to correctly align and interpret transcriptomic information.

### **3.8 RNA Analysis**

#### **3.8.1 RNA Extraction from Root tissue**

##### **Method B TRIZOL Extraction**

Total RNA was extracted from frozen root samples using TRIZOL Reagent (ZYMO RESEARCH) The manufacturer’s (ZYMO RESEARCH) protocol was modified with respect to sample lysis volume, binding conditions, and centrifugation temperature. All centrifugation steps were performed at 13000rpm.

1. Homogenisation: 100mg of frozen root samples was homogenized in 800μl of Trizol reagent by shaking. The homogenate was centrifuged at 13000 rpm for 1 minute at 4°C.
2. Binding: 700 μl was transferred to a new tube. 700 μl of absolute ethanol (100%) was added and mixed thoroughly by inversion. The entire mixture was then transferred to Zymo-Spin IICR Column and centrifuged for 30 seconds. The flow-through was discarded.
3. DNase 1 Digestion
  - The column was washed with 400 μl of RNA Wash Buffer and centrifuged for 30 seconds.
  - DNase 1 Digestion mix was prepared by combining 75 μl of DNA Digestion Buffer with 5μl of DNase 1 enzyme (6U/ μl) in a RNase free tube. The 80 μl mix was added directly to the column matrix.
4. Washing:
  - 400 μl of Direct-zol RNA PreWash was added to the column and centrifuged for 30 seconds at 4°C. This step was repeated for one more time.

- 700 µl of RNA Wash Buffer was added to the column and centrifuged for 30 seconds at 4°. The final centrifugation step of 2 minutes at 4°C was performed with the column empty to ensure complete removal of residual ethanol.
5. Elution: The column was transferred to a clean, RNase-free 1.5 mL tube. To dry the tube, the column cap was opened and left at room temperature for 2-3 minutes to allow any residual ethanol to evaporate. RNA was eluted by applying 50 µl of DNase/RNase-Free Water directly to the column matrix and centrifuged for 1 minute.

### **3.8.2 RNA Quality Assessment**

#### **3.8.2.1 RNA Quantification**

##### Preparation of Standards

Two standards tubes were prepared for fluorometer calibration. Each tube standard, 190 µL of working solution was added to a 0.5 mL assay tube, followed by 10 µL of the respective Qubit RNA BR standard (Standard 1 and Standard 2), for a final volume of 200 µL per tube. Each tube was vortexed for 2-3 seconds and incubated in dark for 2 minutes. Standard 1 represents the lower end of the RNA concentration range, while Standard 2 represents the upper end; these two used to calibrate the Qubit Fluorometer prior to sample measurement.

##### RNA Quantification

For each RNA sample, 199 µL of working solution was added to a 0.5 µL assay tube, followed by 1 µL of the RNA samples, for a total volume of 200 µL. The tube was vortexed for 2-3 seconds and incubated in dark for 2 minutes. RNA concentration was subsequently determined using the Qubit Fluorometer (Thermo Fisher Scientific), selecting the RNA Broader Range assay.

#### **3.8.2.2 Agarose Gel Electrophoresis**

RNA integrity was visualised by electrophoresis through a 1% (w/v) agarose gel prepared as follows.

- 1 g of agarose dissolved in 100 mL of 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3).
- The solution was dissolved in a microwave until the agarose was completely dissolved.

- Cooled to approximately 60°C before being poured into a casting tray with a well-forming comb.

Sample Preparation.

RNA samples: 5 µL of the eluted RNA with 1µL of a 6× DNA loading dye (bromophenol blue and xylene cyanol).

Electrophoresis.

- Gel submerged in 1× TBE buffer in electrophoresis tank.
- Samples loaded into wells alongside molecular weight marker (Invitrogen 1kb Plus DNA Ladder)
- Constant voltage applied 100 V for 30 minutes.

Visualisation.

RNA bands were visualised using the BIO-RAD ChemiDoc MP Imaging System. The presence of distinct 28S and 18S rRNA bands at an approximate 2:1 intensity ratio indicates intact RNA.

### **3.8.3 RNA Seq Preparation**

Total samples passing quality criteria were sent to IGA laboratory in Udine Italy for RNA-seq sequencing.

## **3.9 Statistical Analysis**

All statistical analyses were performed using R version 4.3.2 (R Core Team 2024) with relevant Bioconductor packages, Minitab Statistical Software v22, and Microsoft Office 365 Excel. A significant threshold of  $\alpha = 0.05$  was applied for all statistical tests unless otherwise stated.

### **3.9.1 Data Distribution and Assumption Testing**

Prior to parametric tests, all data were tested for:

Normality: Shapiro-Wilk test ( $p > 0.05$  indicates normal distribution)

Homogeneity of variance: Levene's test ( $p > 0.05$  indicates homogenous variance)

### 3.9.2 Analysis of Colonisation Phenotype (Part 1)

Identification of High/Low Colonising ILs: A one-way (ANOVA) was performed in Minitab 22. For ILs where the ANOVA indicated a significant overall effect ( $p < 0.05$ ), Tukey's honest significant difference (HSD) post-hoc test was used to identify which specific ILs differed significantly from the recurrent parental control (Vialone nano) and from each other.

### 3.9.3 Analysis of Drought Stress Phenotypes (Part 2)

Physiological data from the drought stress experiment (shoot length, relative water content) were analysed using a three-way ANOVA in Minitab with the following factors:

- Factor 1: Genotype (IL189, IL273, Vialone Nano, *Oryza rufipogon*) (4 levels)
- Factor 2: Inoculation (with *K. sacchari* RCA25 vs without ;2 levels)
- Factor 3: Water regime (Control - well watered (100% FC) vs drought stress (50 % FC))

The model included:

- All main effects (IL, Inoculation, water)
- All two-way interactions (IL  $\times$  Inoculation, IL  $\times$  Water, Inoculation  $\times$  Water)
- Three-way interaction (IL  $\times$  Inoculation  $\times$  Water)

Where significant interaction was detected ( $p < 0.05$ ), means were separated using Tukey's HSD post hoc test to identify the source of interaction.

## 4.0 RESULTS

Chapter 4 presents the findings from the three integrated experiments, designed to address the research objective (i) mapping root colonisation patterns of the bacterial endophyte *Kosakonia sacchari* (strain RCA25) among Introgression Lines (ILs) derived crossing *Oryza rufipogon* and *Oryza sativa var. Vialone Nano*; (ii) assessing physiological response to water stress in inoculated and uninoculated lines (IL189, IL273, and their parental lines *Oryza rufipogon* and *Vialone nano*) ; (iii) characterising differential gene expression profiles elucidated through RNA sequencing.

### Section 1: Root Colonisation of Introgression Lines by *Kosakonia sacchari* RCA25

#### 4.1.1 Experimental Design and Quality Control

Initial screening involved inoculation of 41 introgression lines (ILs) and two parental lines (*Oryza rufipogon* and *Vialone nano*) with *K. sacchari* RCA25 using 3 biological replicates and a control per genotype. Bacteria were applied to the seedlings and after 7 days roots were harvested, sterilised, homogenised, serially diluted and plated on medium to quantify *K. sacchari* as CFU per gram of fresh roots. For each genotype, mean CFU/g, standard deviation, and coefficient of variation (CV) across the three biological replicates were calculated, and CFU counts were log<sub>10</sub>-transformed prior to statistical analysis, to stabilise variance and approximate normality (Romano et al., 2020). A complete summary of raw CFU values and descriptive statistics (mean, standard deviation and coefficient of variation) of all 41 ILs and two parental lines is presented in Table 3.

Before hypothesis testing, quality control criteria were applied to ensure that only contamination-free estimates were included in the analysis. Plates from genotypes showing contamination in the mock-inoculated controls were discarded in line with standard microbiological practice that contaminated plates cannot provide reliable quantitative viability data. Genotypes with extremely high variability (CV > 60%) were excluded, as high variability at this level indicate unreliable quantification. By these predefined criteria, 23 genotypes including the parental lines, met the quality threshold and were retained for statistical analysis (Table 4) (Reed et al., 2002)

Table 3 Log<sub>10</sub> -transformed *K. sacchari* (RCA25) root colonisation value (CFU/g fresh root) for 41 ILs and two parental lines, showing means, standard deviations (SD), and coefficient of variation.

Genotype	Log <sub>10</sub> (Mean CFU/g ± SD CFU/g)	Coefficient of Variation (%)
IL10	7.46 ± 0.20	46.49
IL11	8.23 ± 0.44	77.26
IL12	7.29 ± 0.11	26.74
IL13	7.77 ± 0.63	136.37
IL14	7.36 ± 0.19	40.17
IL15	7.68 ± 0.36	58.88
IL16	7.57 ± 0.13	27.44
IL17	7.03 ± 0.11	26.25
IL18	7.66 ± 0.11	23.77
IL189	6.82 ± 0.47	100.50
IL198	7.76 ± 0.07	16.08
IL22	6.96 ± 0.24	44.98
IL23	7.02 ± 0.34	57.30
IL245	7.44 ± 0.26	61.11
IL25	7.70 ± 0.36	59.21
IL26	6.67 ± 0.26	59.13
IL28	8.17 ± 0.08	16.62
IL29	7.75 ± 0.48	112.38
IL30	7.75 ± 0.48	112.38
IL31	7.37 ± 0.16	40.01
IL32	7.30 ± 0.23	58.10
IL34	7.15 ± 0.54	122.82
IL345	7.27 ± 0.07	15.34
IL346	7.87 ± 0.48	69.61
IL36	8.17 ± 0.23	43.02
IL38	7.23 ± 0.22	50.08
IL39	8.39 ± 0.23	43.09
IL4	6.99 ± 0.14	35.03
IL41	7.64 ± 0.13	31.46
IL42	7.26 ± 0.18	35.67
IL43	7.04 ± 0.11	26.75
IL45	6.98 ± 0.66	89.99
IL47	7.55 ± 0.24	45.47
IL50	7.06 ± 0.13	28.65
IL52	6.72 ± 0.08	19.03
IL54	7.65 ± 0.13	28.11
IL6	6.46 ± 0.06	13.98
IL70	7.75 ± 0.81	151.59
IL78	6.08 ± 0.13	32.60
IL8	6.07 ± 0.02	4.22
<i>O. rufipogon</i>	8.23 ± 0.33	56.01
Vialone Nano	7.29 ± 0.20	49.52

Statistical diagnostic procedures verified that the log<sub>10</sub>-transformed CFU data satisfied parameter assumptions: Levene's test indicated homogeneity of variance (F= 0.38, p= 0.988) and Shapiro-Wilk test confirmed normality of residuals (p > 0.100). Subsequent one-way ANOVA showed highly significant variation among the introgression lines (F<sub>20,42</sub> = 22.85, p < 0.001) (Shahid et al., 2022).

Table 4 statistics of *K. Sacchari* CFU counts across 23 genotypes (21 ILs + 2 parental lines).

Parameter	Introgression Line (IL)/ Variety	Mean (CFU/g)	StDev (CFU/g)	CoefVar
CFU/g	IL12	8.83E+07	2.36E+07	26.74
	IL14	2.42E+07	9.71E+06	40.17
	IL15	5.72E+07	3.37E+07	58.88
	IL16	3.79E+07	1.04E+07	27.44
	IL17	1.09E+07	2.87E+06	26.25
	IL18	4.63E+07	1.10E+07	23.77
	IL198	5.75E+07	9.24E+06	16.08
	IL23	1.24E+07	7.09E+06	57.30
	IL25	6.03E+07	3.57E+07	59.21
	IL28	1.50E+08	2.49E+07	16.62
	IL32	2.21E+07	1.28E+07	58.10
	IL345	1.87E+07	2.87E+06	15.34
	IL36	1.61E+08	6.92E+07	43.02
	IL38	1.84E+07	9.21E+06	50.08
	IL41	4.50E+07	1.42E+07	31.46
	IL42	1.91E+07	6.83E+06	35.67
	IL47	3.85E+07	1.75E+07	45.47
	IL50	1.18E+07	3.37E+06	28.65
	IL52	8.83E+07	2.36E+07	19.03
	IL6	2.42E+07	9.71E+06	13.98
	IL8	5.72E+07	3.37E+07	4.22
	<i>O. rufipogon</i>	8.23E+08	1.11E+08	56.01
	Vialone Nano	2.09E+07	1.04E+07	49.52

#### 4.1.2 Root Colonisation of Introgression Lines by *Kosakonia Sacchari* RCA25

A one-way ANOVA revealed significant differences in root colonisation capacity among the 23 Introgression Lines (ILs) ( $F(20, 42) = 22.85, p < 0.001$ ). Post hoc comparison using Tukey's Honestly Significant Difference (HSD) test ( $\alpha = 0.05$ ) showed specific grouping based on mean  $\log_{10}$ CFU/g values (Table 5).

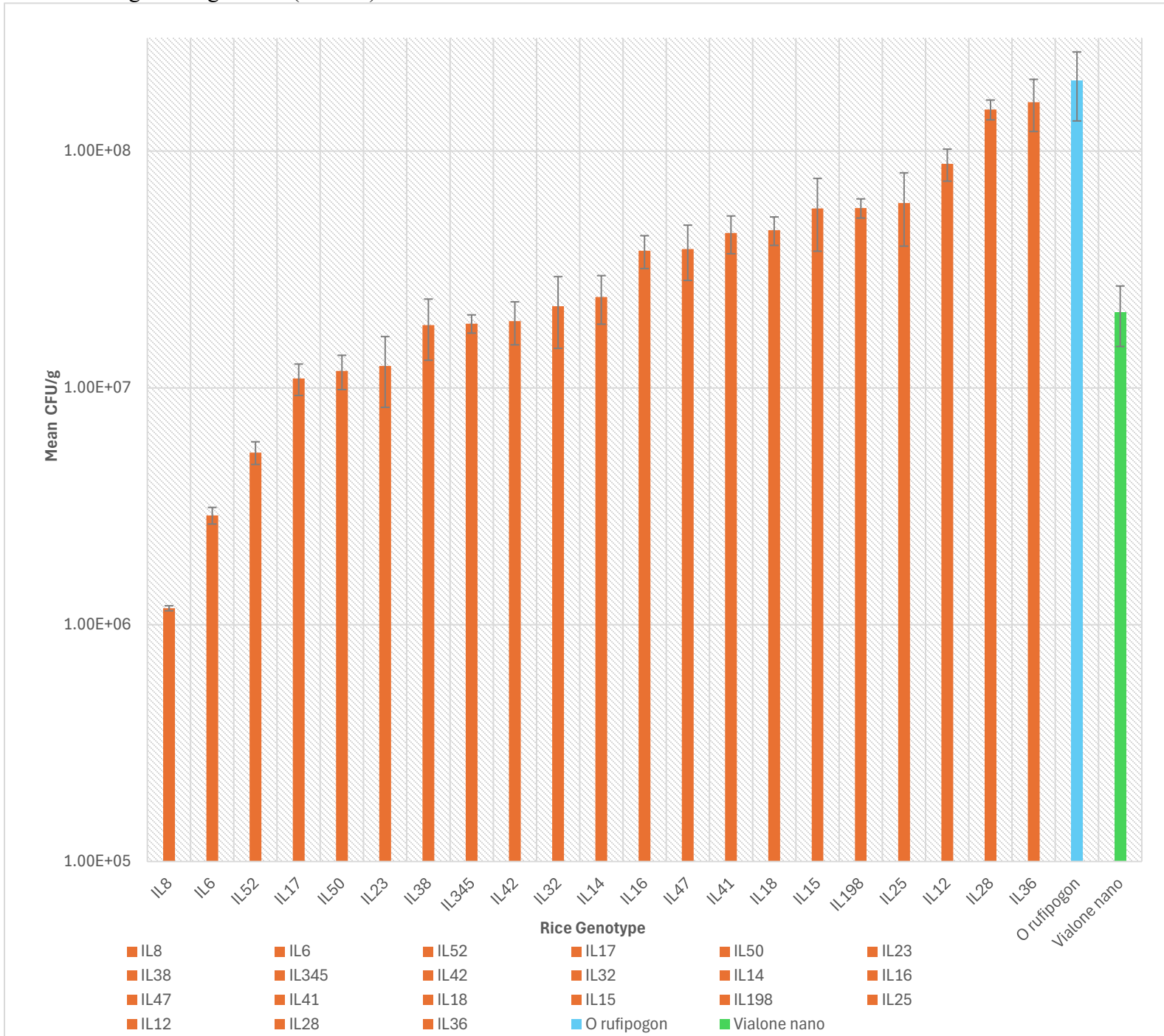


Figure 8 Mean root colonisation levels ( $CFUg^{-1}$  fresh root) of *K. sacchari* RCA25 across 21 introgression lines and two parental lines (*Oryza rufipogon*, blue; *Vialone nano*, green).

Table 5 Means and standard deviation  $\log_{10}$  (CFU/g) for each Introgression Line ILs and parental lines

Introgression Line (IL)/ Variety	Means and standard deviations $\log_{10}$ (CFU/g)
IL36	8.17 ± 0.23 <sup>a</sup>
IL28	8.17 ± 0.08 <sup>a</sup>
IL12	7.94 ± 0.11 <sup>ab</sup>
IL198	7.76 ± 0.07 <sup>abc</sup>
IL25	7.70 ± 0.36 <sup>abc</sup>
IL15	7.68 ± 0.36 <sup>abc</sup>
IL18	7.66 ± 0.10 <sup>abc</sup>
IL41	7.64 ± 0.13 <sup>abcd</sup>
IL16	7.57 ± 0.13 <sup>bcd</sup>
IL47	7.55 ± 0.24 <sup>bcd</sup>
IL14	7.36 ± 0.19 <sup>bcd</sup>
IL32	7.30 ± 0.23 <sup>cd</sup>
IL345	7.27 ± 0.07 <sup>cd</sup>
IL42	7.26 ± 0.18 <sup>cd</sup>
IL38	7.23 ± 0.22 <sup>cd</sup>
IL50	7.06 ± 0.13 <sup>de</sup>
IL17	7.03 ± 0.11 <sup>ef</sup>
IL23	7.02 ± 0.34 <sup>ef</sup>
IL52	6.72 ± 0.08 <sup>fg</sup>
IL6	6.46 ± 0.06 <sup>fg</sup>
IL8	6.07 ± 0.02 <sup>h</sup>
<i>O. rufipogon</i>	8.23±0.33 <sup>a</sup>
Vialone Nano	7.29±0.20 <sup>defg</sup>

*Means that do not share a letter are significantly different from each other*

IL36 exhibited the highest mean colonisation level (8.17  $\log_{10}$ CFU/g) and was statistically superior to all other introgression lines except IL28 (8.17  $\log_{10}$ CFU/g), with which it shared the ‘a’ grouping. In contrast, IL8 demonstrated the lowest colonisation (6.07  $\log_{10}$ CFU/g), assigned to group ‘h’, and was significantly lower than all remaining lines. The hierarchical grouping pattern revealed a continuum of colonisation phenotypes, with ILs sharing common letters representing non-significant differences at the 95% confidence level. These results indicate substantial genetic variation in the ability of introgression lines to support RCA25 root colonisation.

Intermediate values were observed across several (ILs). IL41 (7.64  $\log_{10}$ CFU/g) was assigned to groups ‘a’ through ‘d’, indicating it was not significant different from high-colonising lines but

was significantly lower than lines in the group's 'e', 'f', 'g' and 'h' groups. These grouping letters illustrate a continuum of values, where adjacent groups overlap. Lines such as IL16, IL47, and IL14 are not significantly different from each other but are different from the highest (group a) and the lowest (performers).

## Section 2: Stress Test of IL273, IL189, Vialone Nano and *Oryza rufipogon*

The water stress test was conducted using 4 genotypes: IL189, IL273, Vialone Nano and *Oryza rufipogon*. The two introgression lines (IL189 and IL273) had previously shown contrasting root colonisation efficiency in the initial screen, with IL273 achieving  $2.77 \times 10^8$  CFU g<sup>-1</sup> root fresh weight (CV = 15.27%) and IL189 achieving a mean of  $3.01 \times 10^8$  CFU g<sup>-1</sup> root fresh weight (CV = 29.0%) and. Plants of all the genotypes were either inoculated with *K. sacchari* RCA25 or mock-inoculated (controls) before water stress. Root colonisation (Section 4.2.1), leaf turgidity assessed as relative water content (Section 4.2.2), and shoot length (Section 4.2.3) were quantified, and fresh roots were collected for RNA sequencing to link physiological responses with transcriptional changes (Section 4.3).

### 4.2.1 Root colonisation of the 4 selected genotypes with RCA25

Table 6 Mean and standard deviation of Log (CFU/g) of the phenotypes

Genotype	Means and standard deviation. <i>log<sub>10</sub></i> (CFU/g)
IL189	6.94 ± 0.32 <sup>a</sup>
IL273	6.73 ± 0.12 <sup>a</sup>
<i>Oryza rufipogon</i>	7.11 ± 0.44 <sup>a</sup>
Vialone nano	7,29 ± 0.20 <sup>a</sup>

*Means that do not share a letter are significantly different from each other.*

A one-way ANOVA indicated no significant difference in root colonisation capacity among the four genotypes ( $F(3, 10) = 3.26, p = 0.159$ ) and post-hoc comparison (Tukey's HSD) showed that all group mean shared the superscript "a" confirming that none of the pairwise differences reached statistical significance as shown in Table 6.

Although IL189 exhibited slightly higher mean colonisation level (6.94 log<sub>10</sub>CFU/g) than IL273 (6.73 log<sub>10</sub>CFU/g), this difference was not statistically significant. Notably *O. rufipogon* had slightly lower colonisation mean (7.11 log<sub>10</sub>CFU/g), than to Vialone Nano (7.29 log<sub>10</sub>CFU/g), though neither difference reached statistical significance. Despite the numerical trend, no genotype exhibited statistically higher colonisation than any other. Figure 9 bar chart illustrates these trends in colonisation across the four genotypes.

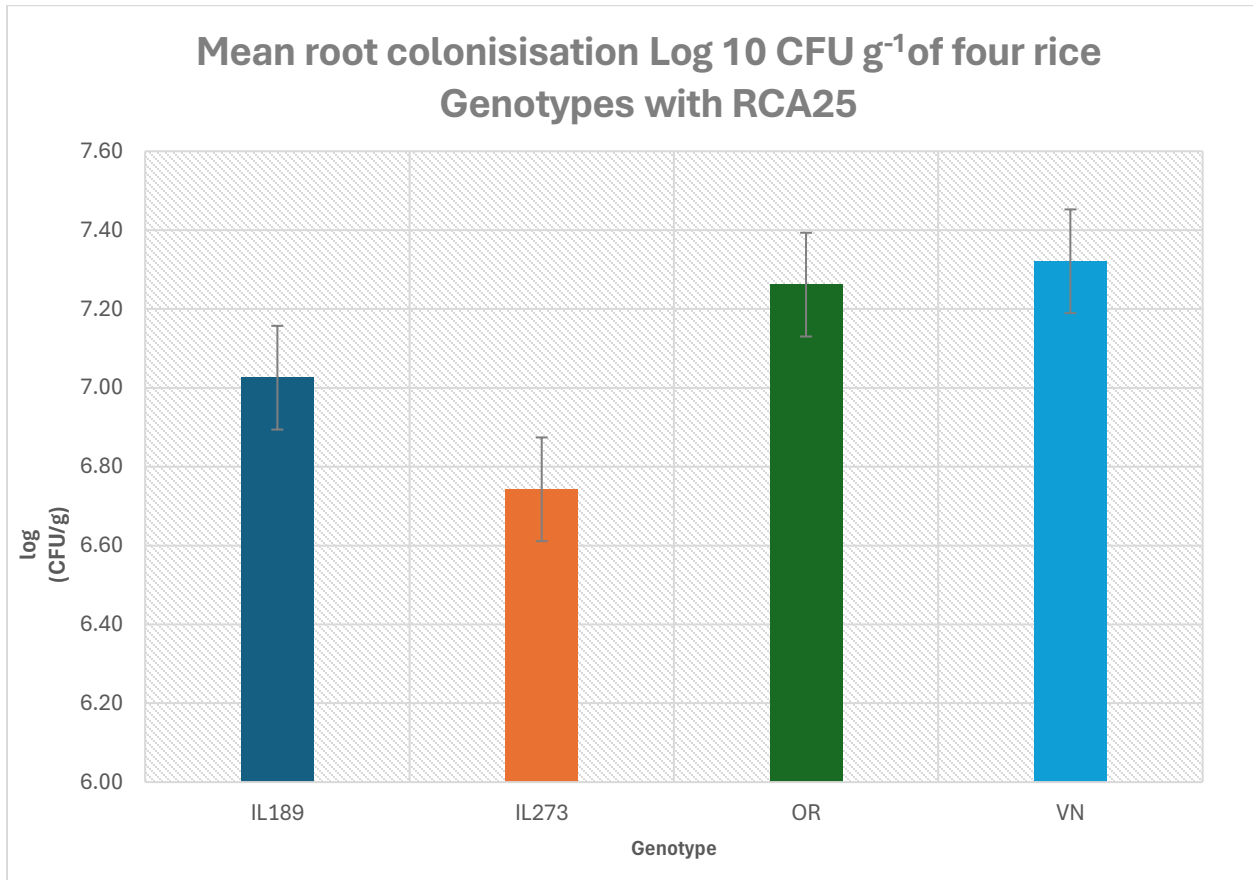


Figure 9 Root colonisation log<sub>10</sub> CFU g<sup>-1</sup> of four rice genotypes.

#### 4.2.2 Relative Water Content (RWC) Analysis

Relative water content (RWC) was measured to evaluate the physiological water status of the four different rice genotypes subjected to *K. sacchari* inoculation under varying water regimes varying water conditions. For each genotype, six pots were assigned and RWC was determined using three leaf samples per pot, giving a total of 144 measurements (4 genotypes × 2 treatment × 2 water levels × 3 pots × 3 leaves per pot). RWC was calculated using the formula:

$$\text{RWC}(\%) = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

Where FW =fresh weight, TW =turgid weight and DW = dry weight. Descriptive statistics of RWC for all the treatments (50% and 100% of water; inoculated/control) and all the genotypes (IL189, Il 273, *O.rufipogon*, Vialone Nano) are summarised in Table 7. Figure 10-13 shows Bar chart of the Relative water content of each genotype.

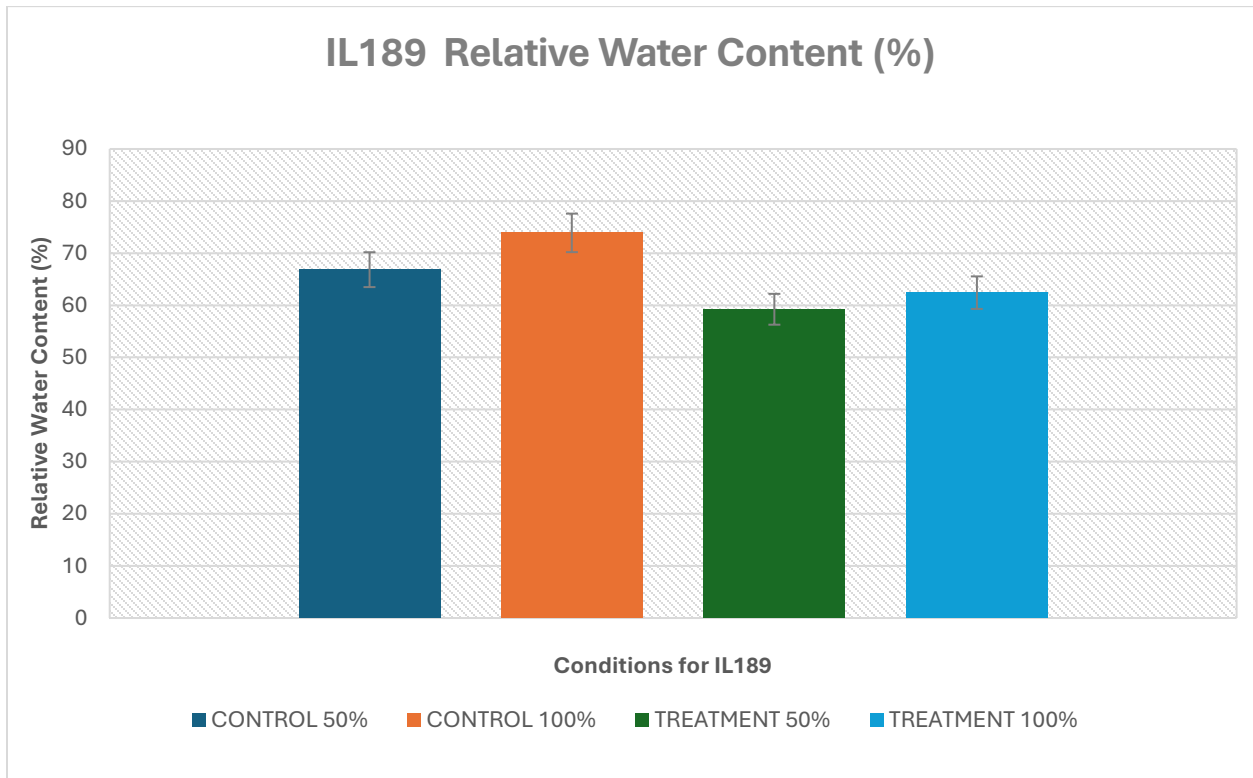


Figure 10 Relative Water Content (%) of IL189

IL189, the untreated control at 100% recorded the highest RWC (73.9%), followed by the control at 50% FC(66.8%). Inoculated plants showed lower RWC at both water levels (59.2% at 50% FC and 62.4% at 100% FC), with the treated 50% FC group recording the lowest overall. As observed for shoot length, inoculation was associated with reduced leaf water status in this genotype regardless of water regime.(Figure 10).

IL273, RWC values were comparatively similar across all treatment groups, ranging from 66.4% to 71.1%.The treated 100% FC plants recorded the highest RWC(71.1%), closely followed by the control 50% FC group (70.4%).The control 100% FC groups recorded the lowest RWC (66.4%).The narrow range of values suggest that neither inoculation nor water deficit imposed a strong differential effect on leaf water status in this genotype (Figure 12).

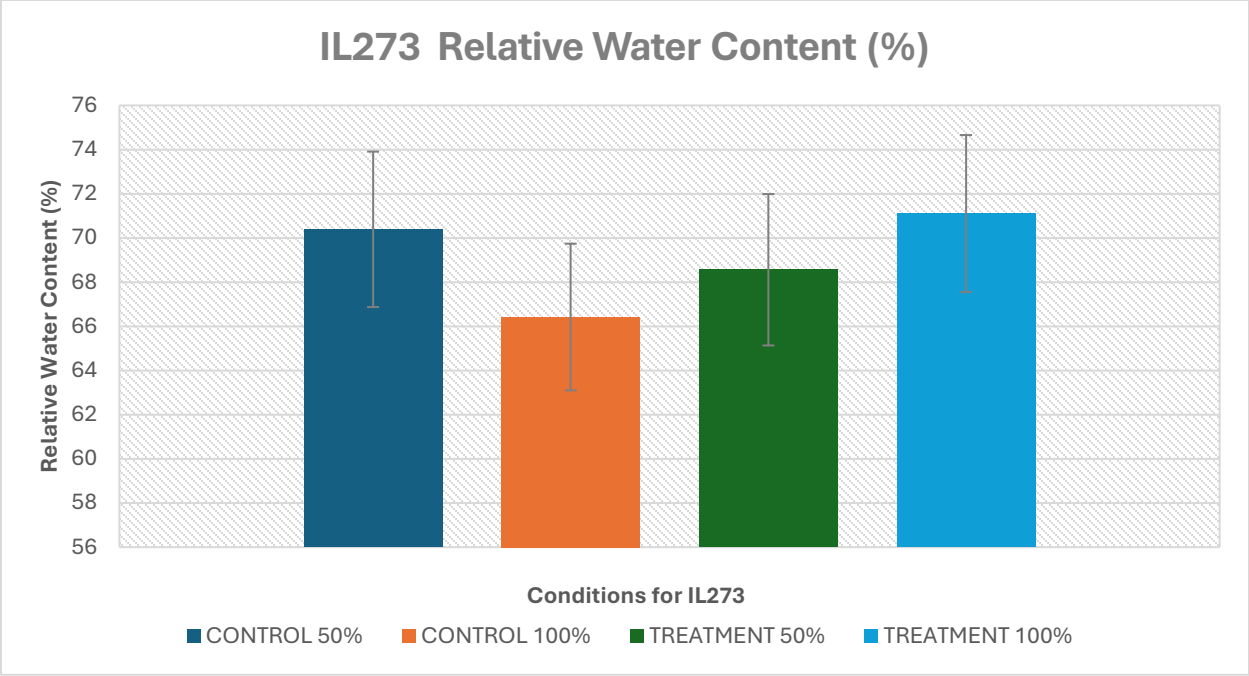


Figure 11 Relative Water Content (%) of IL273

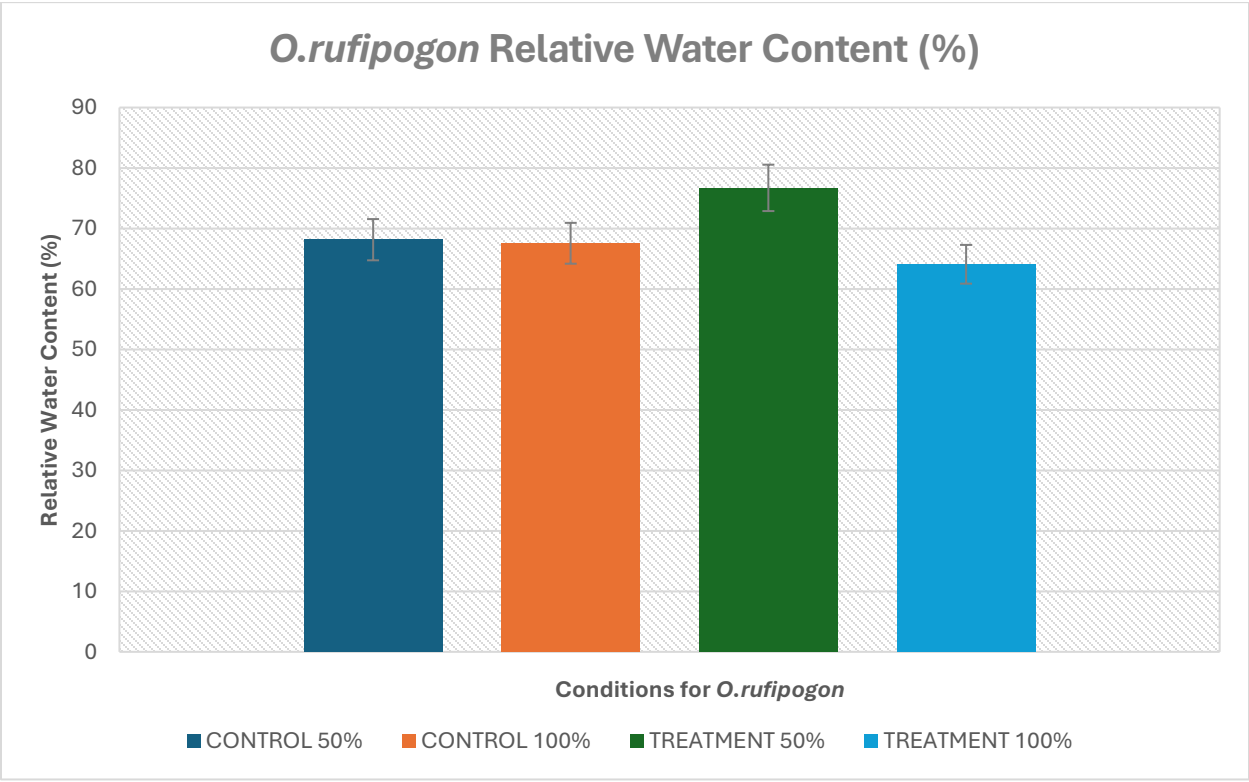


Figure 12 Relative Water Content (%) of *O.rufipogon*

In *O.rufipogon*, a divergent pattern was observed. Inoculated plants at 50% FC recorded the highest RWC (76.7%), markedly exceeding all other groups, while inoculated plants at 100% FC showed the lowest RWC (64.1%). Both control groups were intermediate and similar to each other (67.6-68.1%). The contrasting responses between two inoculated water suggest that the interaction between inoculation and water availability in this genotype is not straightforward (Figure 12)

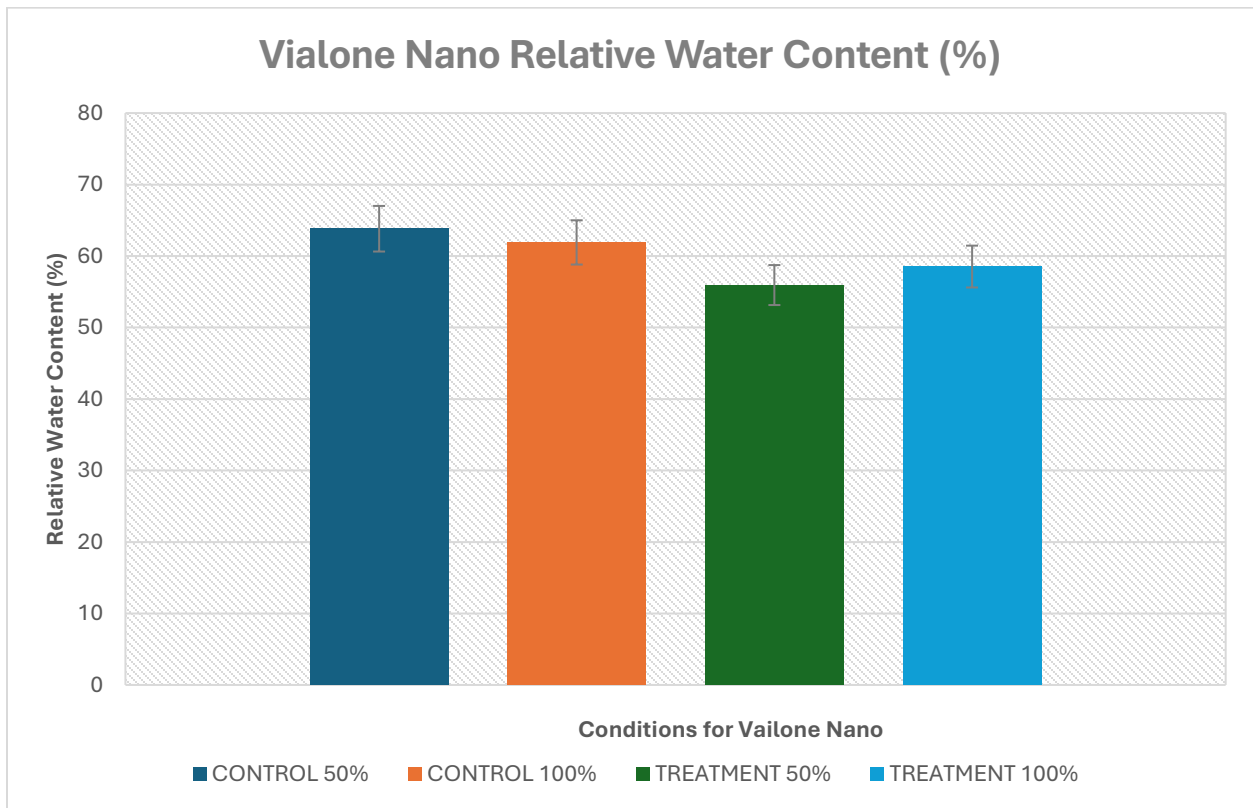


Figure 13 Relative Water Content (%) of Vialone Nano

In Vialone Nano, uninoculated control plants recorded higher RWC than inoculated plants under both water regimes. The control at 50% FC showed the highest RWC (63.8%), followed closely by the control at 100% FC (61.9%). Inoculated plants at both water levels recorded lower RWC values (55.9% at 50% FC and 58.5% at 100% FC), with the treated 50% FC group recording the lowest overall. This pattern is consistent with the trend observed in IL189 and IL273, where inoculation was associated with reduced water status relative to uninoculated control regardless of water availability (Figure 13).

Overall inoculation with *K.sacchari* RCA25 did not produce uniform effects on relative water content across genotypes or water regimes, the significance of these observed differences was further examined using a three-way General Linear Model (see Section 4.2.2.1)

Table 7 Mean Relative Water Content % for Four Rice Genotypes under different inoculation and water level conditions.

Genotype	Water Level (FC)	RWC Control	RWC Treated
		( <i>Non-inoculated</i> )	( <i>Inoculated</i> )
		Mean ± SD	Mean ± SD
IL189	50%	66.84 ± 6.59	59.25 ± 17.54
IL189	100%	73.90 ± 4.83	62.43 ± 7.98
IL273	50%	70.39 ± 9.28	68.56 ± 6.71
IL273	100%	66.42 ± 6.22	71.10 ± 5.69
<i>O. rufipogon</i>	50%	68.15 ± 17.23	76.72 ± 11.46
<i>O. rufipogon</i>	100%	67.56 ± 11.92	64.09 ± 11.61
<i>V.Nano</i>	50%	63.82 ± 8.54	55.94 ± 4.94
<i>V.Nano</i>	100%	61.90 ± 5.76	58.53 ± 3.80

#### 4.2.2.1 Main Effects and Lack of Fit

The data were subsequently analysed using a General Linear Model (GLM) with three fixed factors. The summary for the ANOVA is presented in Table 8.

Table 8 Three-way ANOVA results for the effects of Treatment (Inoculation), Water Level and Genotype on Relative Water Content (RWC).

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Genotype	3	698.82	232.94	2.65	0.059
Treatment	1	123.96	123.96	1.41	0.241
Water Level	1	0.11	0.11	0.00	0.972
Error	50	4402.37	88.05		

Lack-of-Fit	10	782.04	78.20	0.86	0.573
Pure Error	40	3620.33	90.51		
Total	55	5225.26			

The three-way GLM ANOVA (Table 8) revealed that neither Treatment (Inoculation  $P= 0.241$ ) nor the Water level ( $P=0.972$ ) had a statistically significant main effect on RWC. At  $\alpha=0.05$  level the effect of Genotype was not statistically significance ( $F (3,50) = 2.65 P=0.059$ ). The non-significant Lack of Fit test ( $P=0.573$ ) confirmed that the model provided an adequate fit for the data.

#### Post-Hoc Comparison of Genotypes

The Post hoc was consistent with the ANOVA, the Tukey's HSD pairwise comparisons grouped all the genotypes into a single statistical group.

Table 9 Tukey grouping and mean of Relative Water Content (RWC)% for Rice Genotypes

Genotype	Mean RWC (%)
IL273	69.12 <sup>a</sup>
<i>O. rufipogon</i>	69.13 <sup>a</sup>
IL189	65.60 <sup>a</sup>
V. nano	60.05 <sup>a</sup>

*Mean sharing the same letter are not significant different (P <0.05).*

#### 4.2.3 Shoot Length Analysis

Shoot length was measured after one week of water stress treatment for all four genotypes across four conditions: uninoculated control at 50% field capacity (FC), uninoculated control at 100% FC, *K. sacchari*-inoculated at 50% FC and *K. sacchari* inoculated at 100% FC. A total of 264 leaves were recorded, with the number of replicates of ranging from 12 to 21 depending on genotype. Results are presented in Figure 10 to 13.

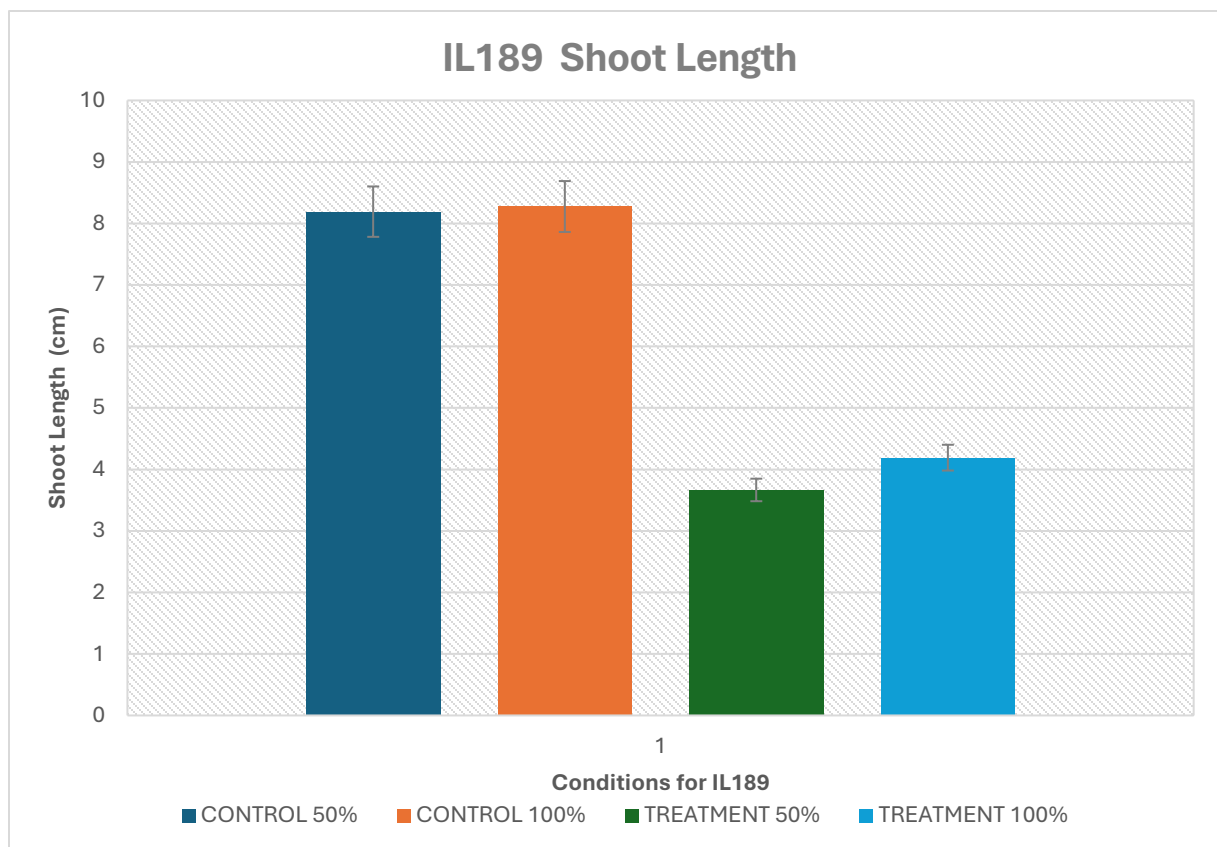


Figure 14 length of IL189 after one week of water stress

IL189, controls at both water levels produced longer shoots (8.2-8.3 cm) compared with treated plants (3.4-4.2cm). The treated 50% FC plant showed the shortest shoot overall, indicating that inoculation was associated with reduced shoot elongation in this line under both water regimes, with no apparent protective effects of the bacterium under drought (Figure 14).

IL273 showed the same general trend as in IL189 (Figure 15). The untreated controls outperformed the treated plants at both water levels, with the 100% FC control recording the greatest shoot length (10.9 cm) and the 50% FC control also performing well (8.5 cm). Inoculated plants at 50% showed the shortest shoots (5.9cm), followed by treated plants at 100 FC (6.8 cm). As with IL189, inoculation did not mitigate the effect of water deficit and appeared to suppress growth relative to uninoculated controls (Figure 11).

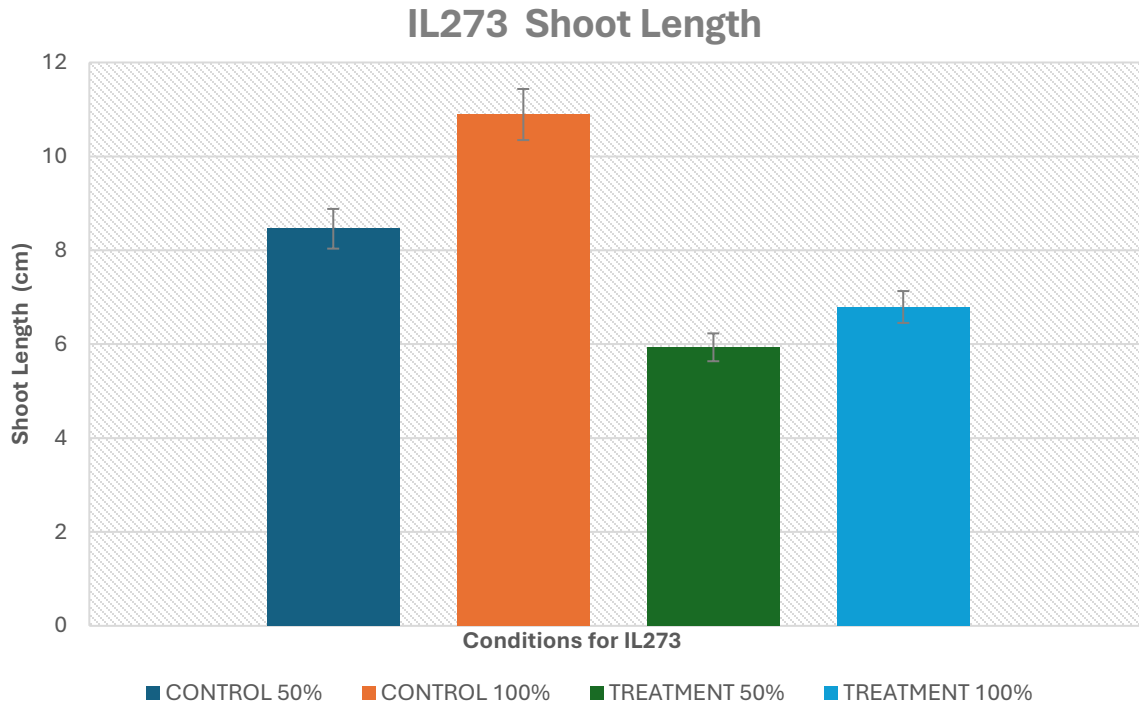


Figure 15 Shoot length of IL273 after one week of water stress.

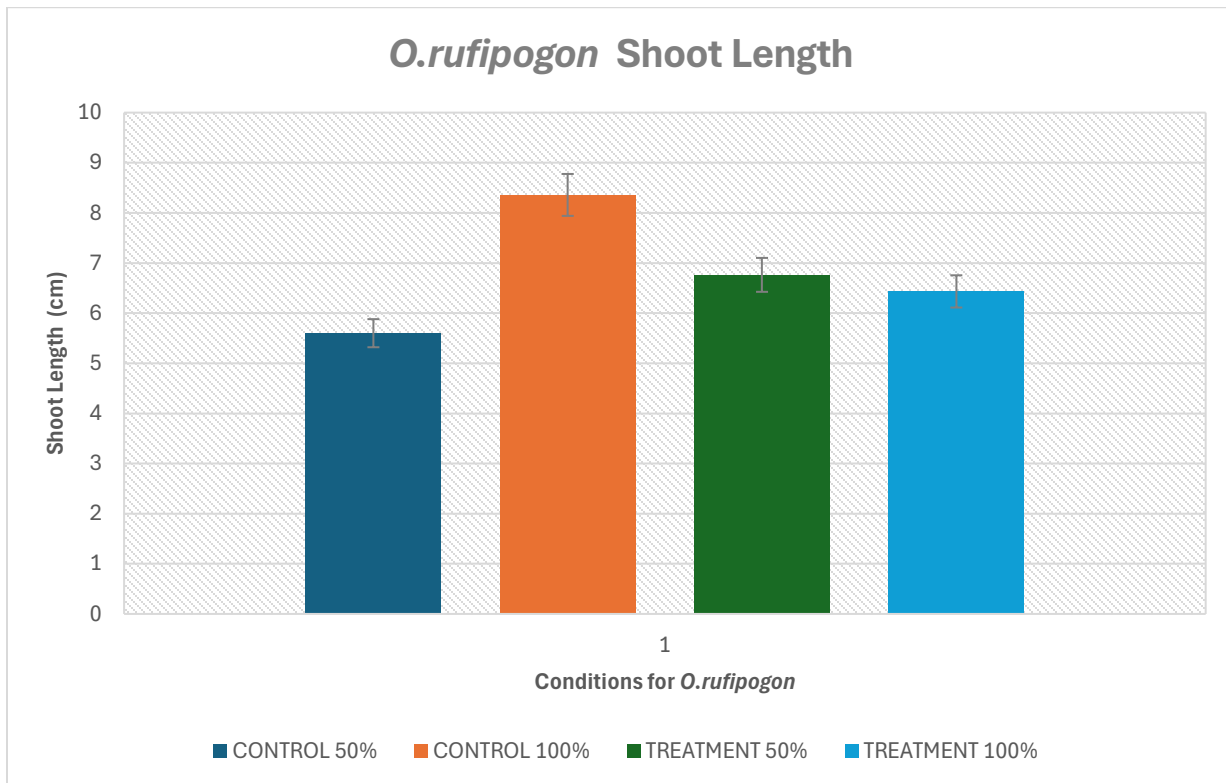


Figure 16 Shoot length of *Oryza rufipogon* after one week of water stress.

In *O. rufipogon*, untreated (controls) plants at 100% FC achieved the greatest shoot elongation (8.3cm). Inoculated plants at both water regimes performed similarly to each other (6.4-6.7cm), while the untreated control at 50% FC recorded the lowest shoot length (5.6cm). Overall, inoculation did not improve shoot length and slightly reduced shoot length relative to the well-watered uninoculated control (Figure 16).

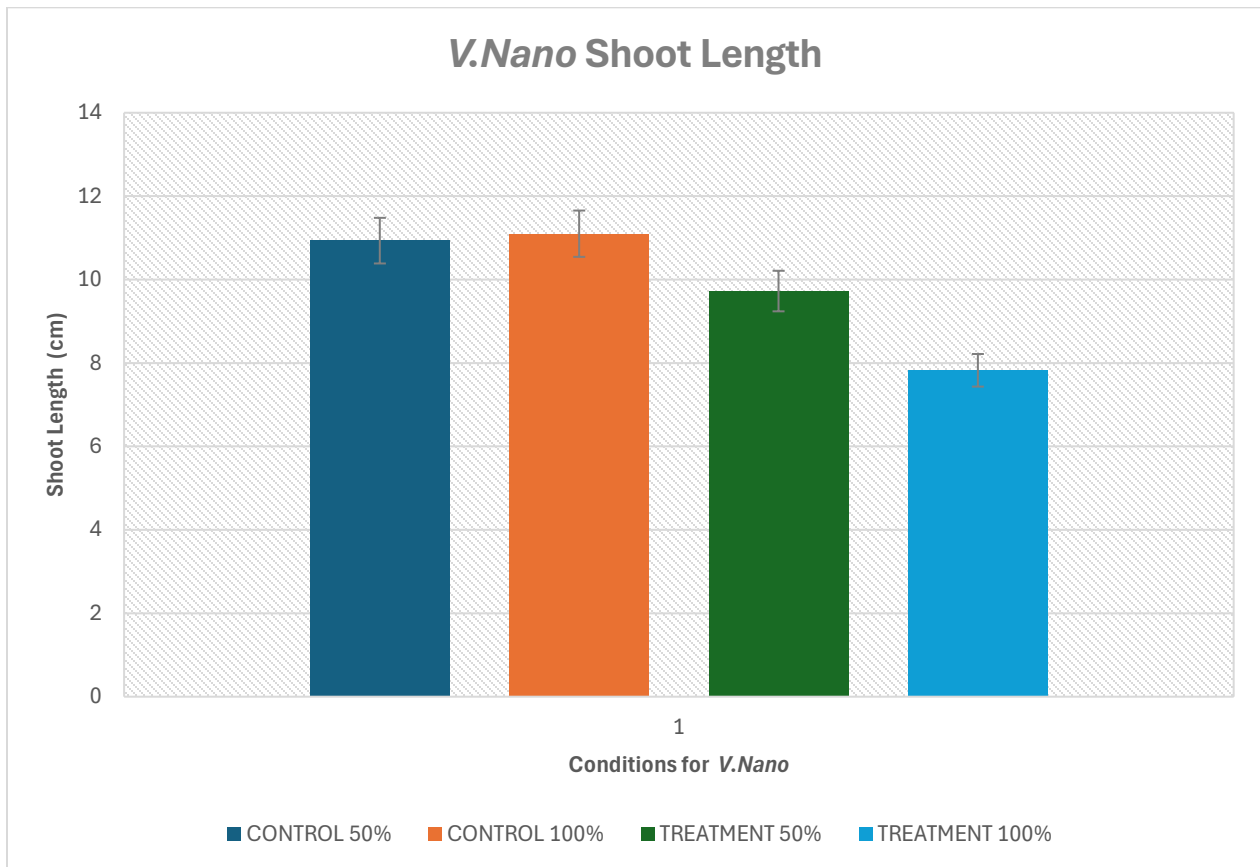


Figure 17 Shoot length of Vialone Nano after one week of water stress.

In Vialone Nano (Figure17), treated plants consistently produced longer shoots than untreated (controls) under both water regimes. The highest shoot length was record in treated plants at 100% (4.6 cm), followed by inoculated plants at 50% FC (3.8 cm), while both control groups recorded the shortest shoots (2.2cm), with slight difference between 50% and 100% FC controls. This was the only genotypes in which inoculation conferred a positive effect on shoot elongation regardless of water availability.

Across the four genotypes, shoot length responses to inoculation was clearly genotype-dependent. Vialone Nano was the only genotype in which inoculated plants outperformed untreated (controls),

while in IL189, IL273 and *O. rufipogon*, control plants consistently achieved greater elongation than treated plants.

#### 4.2.3.1 Main Effects and Interactions

To test whether the shoot length observed differences were statistically significant, shoot length data were analysed using a three-way General Linear Model (GLM) with Genotype, Treatment (inoculated with *K.sacchari* RCA25) and Water Levels as fixed factors (Table 10).

Table 10 Three-way General Linear Model (GLM) Analysis of Variance for shoot length (cm) of four rice genotype (*Oryza sativa* var Vialone Nano, IL189, IL273 and *O.rufipogon* ) subjected to inoculation with *Kosakonia sacchari* RCA25 and two water regimes (50% and 100% field capacity). Significant effects ( $p < 0.05$ ) are shown in bold

Source	DF (Degree of Freedom)	Adj SS (Adjusted Sum of Squares)	Adj MS (Adjusted Mean Square)	F-Value	P-Value
Genotype	3	525.96	191.99	23.93	<b>0.000</b>
Treatment	1	574.97	574.97	71.67	<b>0.000</b>
Water Level	1	13.63	13.63	1.70	0.194
Genotype × Treatment	3	98.66	32.89	4.10	<b>0.007</b>
Genotype × Water Level	3	37.62	12.54	1.56	0.199
Treatment × Water Level	1	31.67	31.67	3.95	<b>0.048</b>
Genotype × Treatment × Water Level	3	29.13	9.71	1.21	0.307
Error	241	1933.39	8.01		
Total	256	3285.63			

*Significant effects ( $p < 0.05$ ) are shown in bold .*

Both Genotype ( $F(3,241) = 23.93, p < 0.001$ ) and Treatment ( $F(1,241) = 71.67, p < 0.001$ ) were highly significant main effects. Water Level alone did not produce a significant main effect on shoot length ( $F(1,241) = 1.70, p = 0.194$ ), indicating that moderate drought (50% FC) did not systematically reduce shoot elongation across all genotypes within the one-week experimental window.

The Genotype  $\times$  Treatment interaction was significant ( $F(3,241) = 4.10, p=0.007$ ), confirming that the effect of *K.sacchari* inoculation on shoot length was not a uniform across genotypes, a result consistent with the contrasting patterns visible in Figure 14-17. A significant Treatment  $\times$  Water Level interaction was also detected ( $F(1,241) = 3.95, p= 0.048$ ), indicating that the relationship between water availability and inoculation effect differed depending on treatment. The three-way interaction (Genotype  $\times$  Treatment  $\times$  Water Level) was not significant ( $p = 0.307$ ), suggesting that the Genotype  $\times$  Treatment effect did not vary strongly between water regimes.

### **4.3 RNA Extraction**

Total RNA was extracted from root tissue samples collected from the four genotypes: IL273, IL189, Vialone Nano and *Oryza rufipogon* yielding a total of 48 samples. RNA extraction was performed using the protocol described in section 3.8.1, which included a liquid nitrogen grinding step, followed by column-based extraction.

Two extraction batches were carried out in this study. Batch 1 refers to RNA extracted from the first water stress experiment, comprising two extraction sets: a first extraction set carried out on 3 November 2025, and a second extraction set carried out on 9 February 2026 using an optimised protocol on the same Batch 1 plant material. Batch 2 refers to RNA extracted from a repeated water stress experiment conducted specifically for the parental lines Vialone Nano and *O.rufipogon*, whose Batch 1 samples showed extensive degradation. Batch 2 samples were extracted on 9 February 2026 from freshly harvested root tissue collected during the repeated experiment.

#### **4.3.1 RNA Yield and Quantification**

For every extraction, Qubit 4 Fluorometer was used to quantify RNA in all samples. Resulting RNA yield is expressed as concentration in ng/uL. Sample of superior quality, as assayed in section 4.3.2 were submitted to RNA sequencing facility, at a minimum of 20 ng/uL in a volume of 10  $\mu$ L, as summarised in Table 11.

Table 11 RNA concentration and volumes after the extractions

<i>Sample</i>	<i>Concentration ng/<math>\mu</math>L</i>	<i>Total Volume <math>\mu</math>L</i>	<i>Sample</i>	<i>Concentration ng/<math>\mu</math>L</i>	<i>Total Volume <math>\mu</math>L</i>
IL 273 C1 50%	23.4	33	IL 273 T1 50%	98.4	89
IL 273 C2 50%	21.7	57	IL 273 T2 50%	71.6	43
IL 273 C3 50%	23.7	44	IL 273 T3 50%	99	33
IL 273 C5 100%	23.6	22	IL 273 T5 100%	73.4	29
IL 273 C6 100%	27.2	29	IL 273 T6 100%	68	44
IL 273 C7 100%	20.6	49	IL 273 T7 100%	46.2	56
IL 189 C1 50%	73.6	77	IL 189 T1 50%	104	54
IL 189 C2 50%	80.6	72	IL 189 T2 50%	43.8	34
IL 189 C3 50%	56.2	90	IL 189 T3 50%	181	52
IL 189 C5 100%	43.8	56	IL 189 T5 100%	33.4	53
IL 189 C6 100%	75.2	40	IL 189 T6 100%	128	47
IL 189 C7 100%	75.4	43	IL 189 T7 100%	42	33
VN C1 50%	73.4	38.8	VN T1 50%	232	18.2
VN C2 50%	28.4	50	VN T2 50%	224	29
VN C3 50%	150	40	VN T3 50%	132	15.4
VN C4 100%	29.2	50	VN T5 50%	240	27.6

VN C5 100%	31.2	50	VN T7 50%	134	35
VN C6 100%	50.8	50	VN T1 50%	77.33	27
OR C1 50%	126	30.6	OR T101 50%	46.8	40
OR C2(2) 50%	44	40	OR T2 50%	59	34.6
OR C3 50%	107	31.6	OR T3 50%	32	40
OR C4 100%	110	38	OR T4 100%	27.8	40
OR C5 100%	86.8	31.8	OR T5 100%	17.2	20
OR C6 100%	67.8	23.8	OR T6 100%	39.8	40

#### 4.3.2 RNA Integrity Assessment by Agarose Gel Electrophoresis

RNA quality was assessed by 1% agarose gel electrophoresis. Total RNA migrates as two distinct ribosomal RNA bands: the 28S rRNA (upper band) and 18S rRNA (lower band), with an expected intensity ratio of 2:1. A faint band near the bottom of the gel shows the migration of small RNAs, mRNA and other small-sized molecules and is not indicative of degradation. The absence of high molecular weight smearing above the 28S band confirms the absence of genomic DNA contamination. Smearing below 18S band indicates partial or complete RNA degradation. The gel images were interpreted as described below.

Prior to the main extractions, a series of trial extractions were carried out on a subset of samples to determine the minimum starting material required for reliable RNA recovery and to optimise lysis conditions. This trial lane is visible in Figure 19; the reduced band intensity in this lane reflects the lower input material (17 mg) used and does not indicate a quality problem with the protocol.

High-quality samples (Figure 18, 19,22): RNA extracted from IL273 and IL189, both control and treated- consistently produced clear 28S and 18S rRNA in expected 2:1 intensity ratio, with no DNA contamination above the 28S band and only faint small RNA bands near the bottom of the

gel. Vialone Nano control samples (VN C1-C7) from Batch 1 and the majority of *O.rufipogon* and Vialone Nano samples from Batch 2 also met these quality criteria. All samples shown in Figure 22 displayed good integrity and were considered suitable for RNA-seq.

Degraded samples: Batch 1 parental lines (Figure 20,21,24): *O. rufipogon* control samples from Batch 1 showed markedly variable and in several cases severely compromised RNA integrity. OR C1 displayed no visible rRNA bands. OR C2 and OR C7 showed extensive smearing below the 18S bands with no distinct bands, consistent with near-complete degradation. OR C3 exhibited faint bands migrating below the expected 18S position, indicating partial degradation. OR C5 showed a visible but reduced 28S band with smearing below 18Sm suggesting incomplete preservation. Vialone Nano treated samples (VN T1-T6) in Batch 1 similarly displayed smearing below 18S, with OR C7 being the most severely affected. No DNA contamination was detected the 28S band above in any lane. These results confirmed the need for re-extraction of all parental line samples.

Batch 1 re-extraction results (Figure 23): Direct comparison of OR C2 and OR C7 between batches confirmed the improvements achieved by protocol optimisation-Batch 1 samples showed extensive degradation with no distinct bands, while the same samples re-extracted in Batch 2 displayed intact 28S and 18S rRNA bands in the correct ratio. Overall, all Batch 2 *O.rufipogon* and Vialone Nano samples met the RNA integrity threshold required for downstream RNA-seq library preparation.

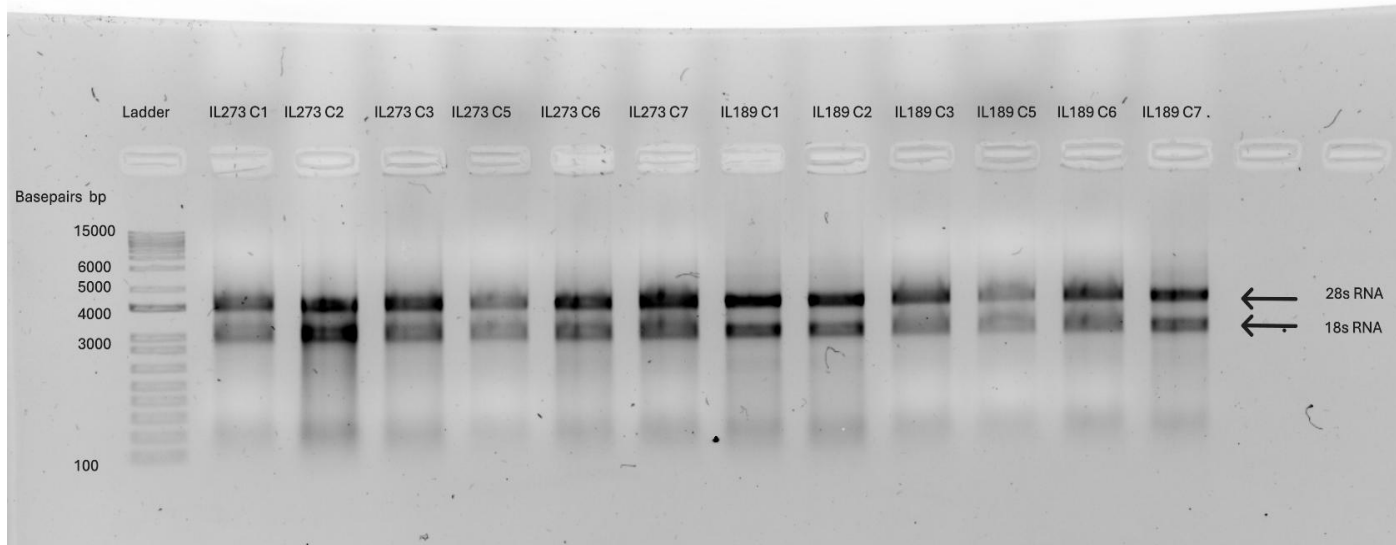


Figure 18: RNA integrity assessment by agarose gel electrophoresis

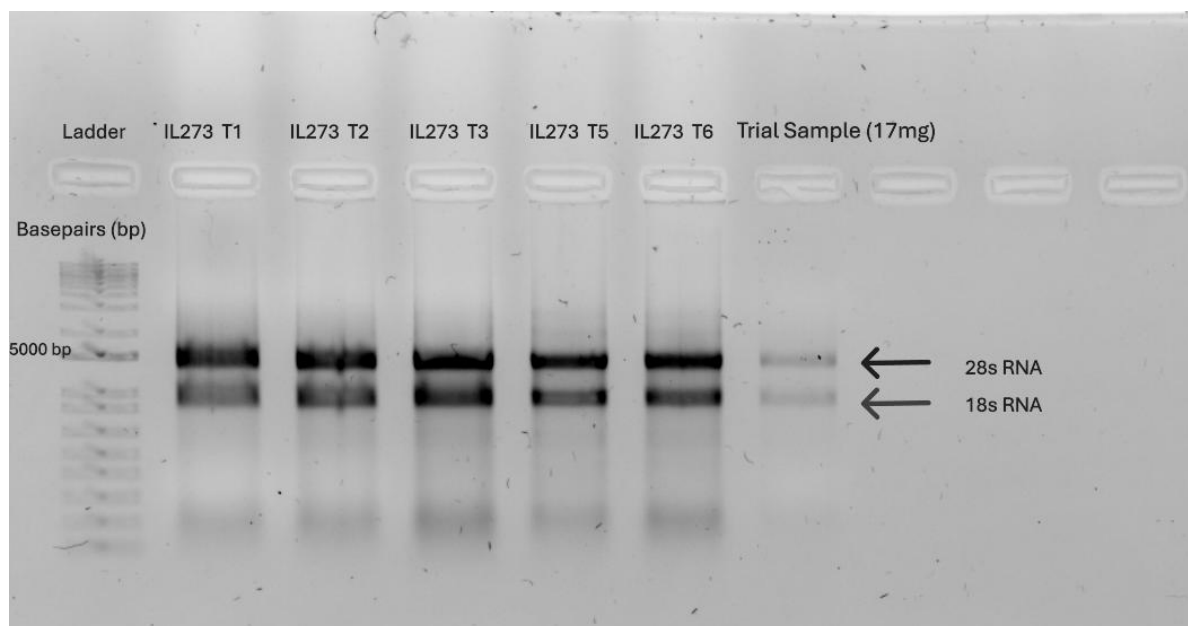


Figure 19: RNA integrity of IL273 samples and trial extraction.

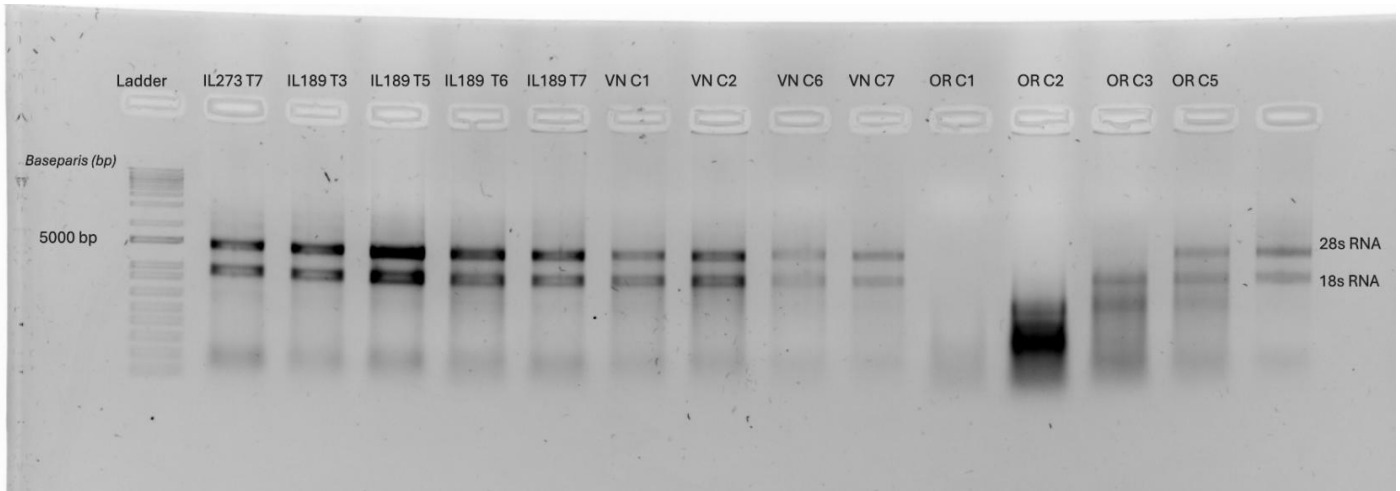


Figure 20: RNA profiles across IL273, IL189, OR and OR treatments.

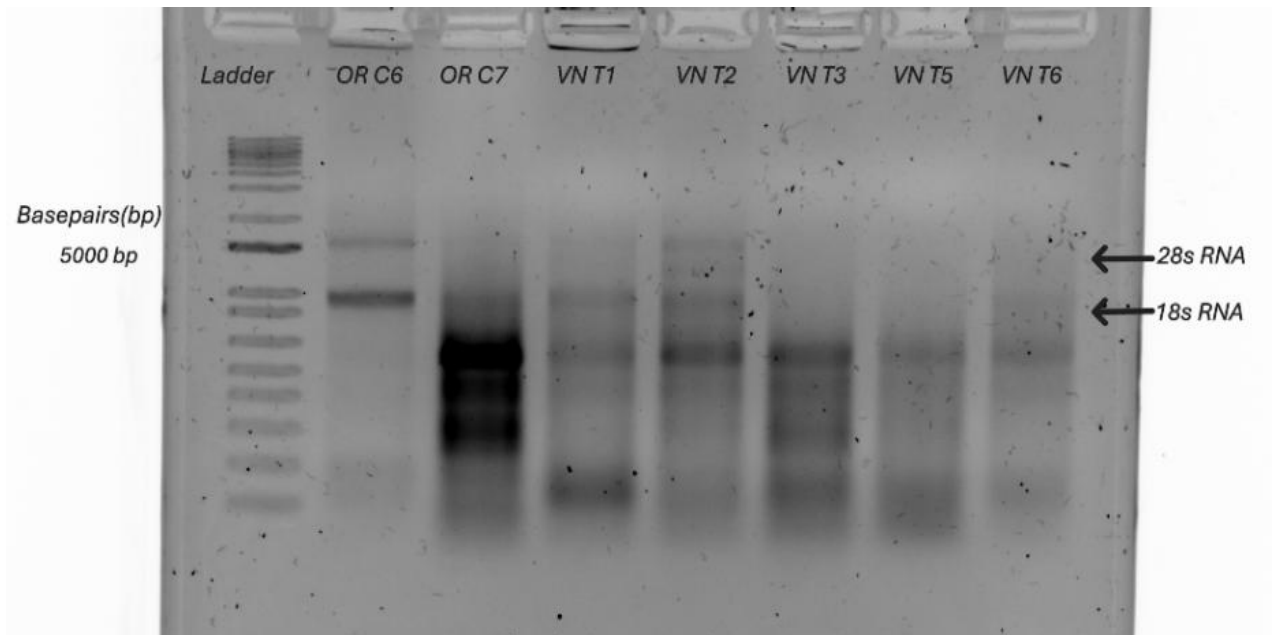


Figure 21: RNA quality in OR and VN treatment samples.

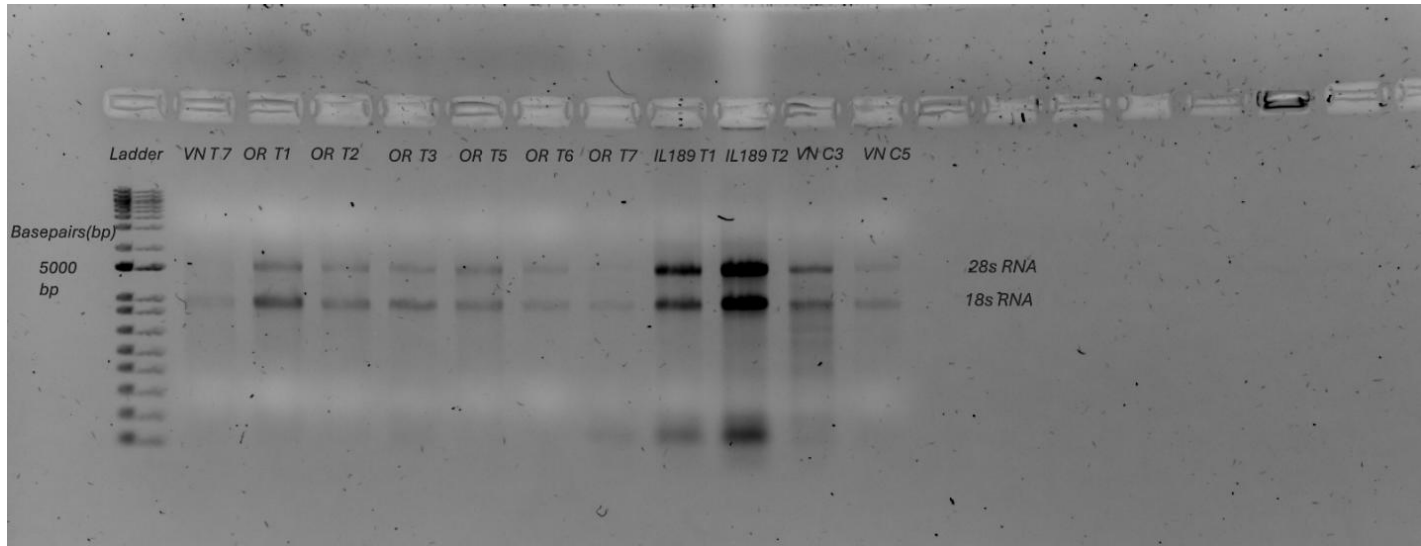


Figure 22 RNA integrity in VN, OR and 189 samples.

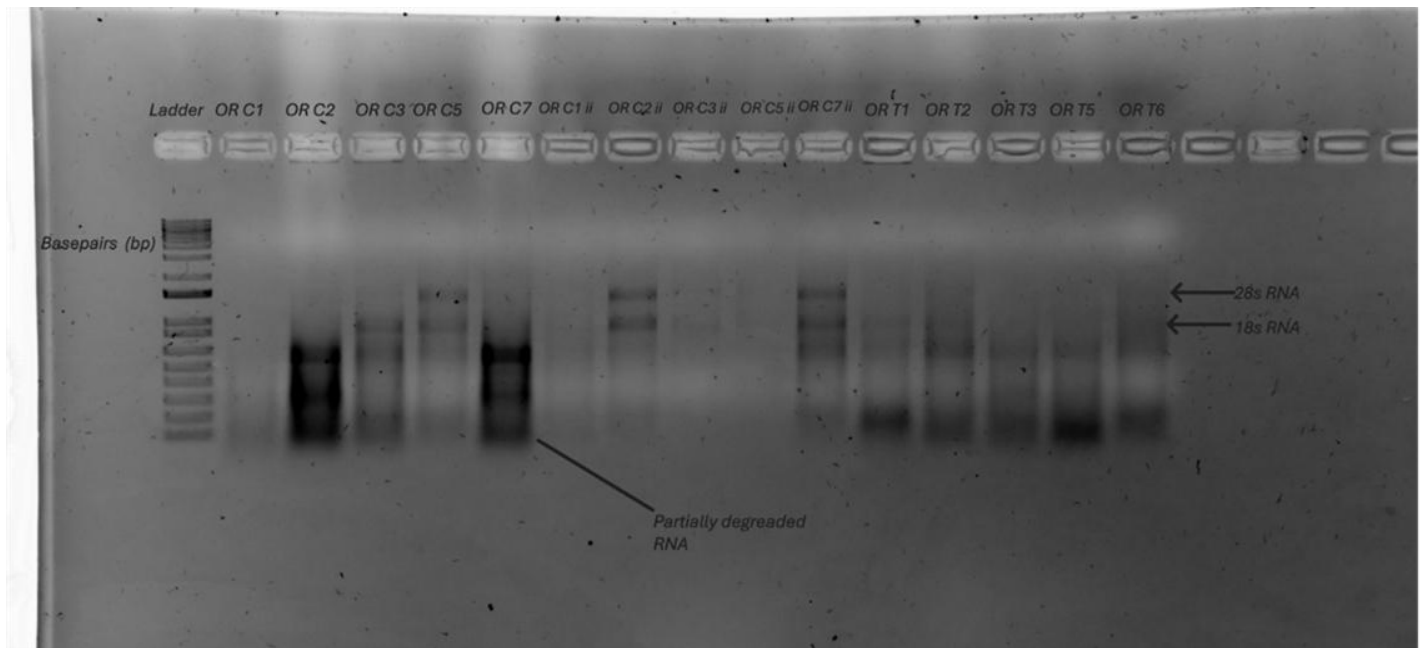


Figure 23 RNA integrity in OR replicates.

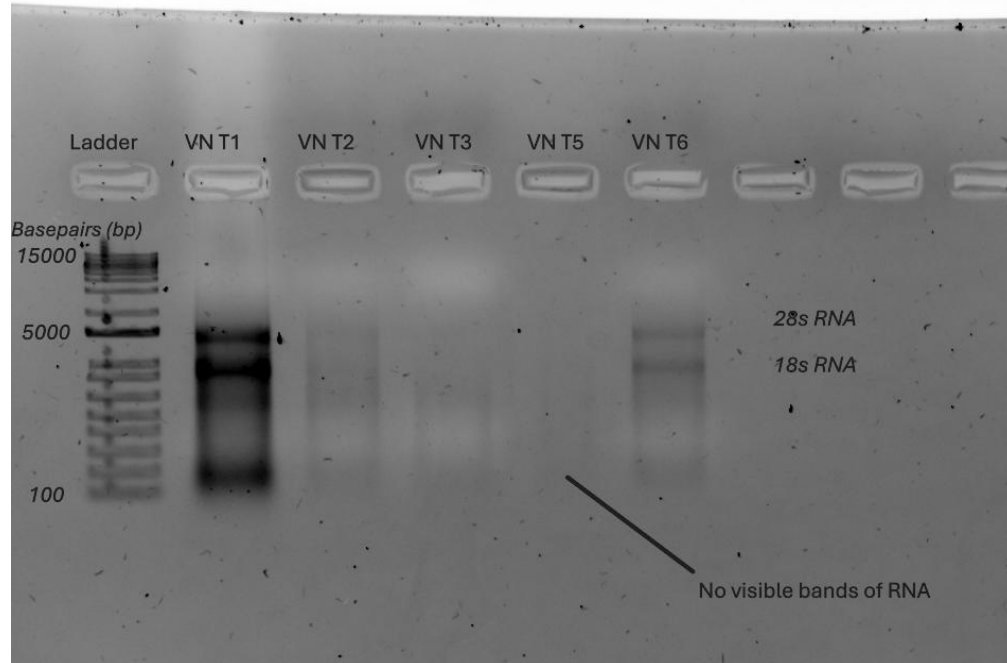


Figure 24: RNA integrity across Vialone Nano treated samples.

To obtain high-quality RNA quality from the parental line, the water stress experiment was repeated specifically for Vialone Nano and *O. Rufipogon*. RNA was extracted from these freshly harvested samples on 9 February 2026, constituting Batch 2.

RNA extracted from parental lines samples (OR C1-C6, OR T1-T7) and VN C1-C6 showed clear 28S and 18S rRNA bands across all lanes, indicating good RNA integrity (Figure 25). Faint bands near the bottom of the gel, visible in several lines, represent small RNAs and minor degradation and do not compromise the sample quality. No DNA contamination was observed. All samples in this subset were considered of excellent quality for downstream analyses. The VN T1-T6 showed some form of degradation, and the RNA extraction was repeated (Figure 26).

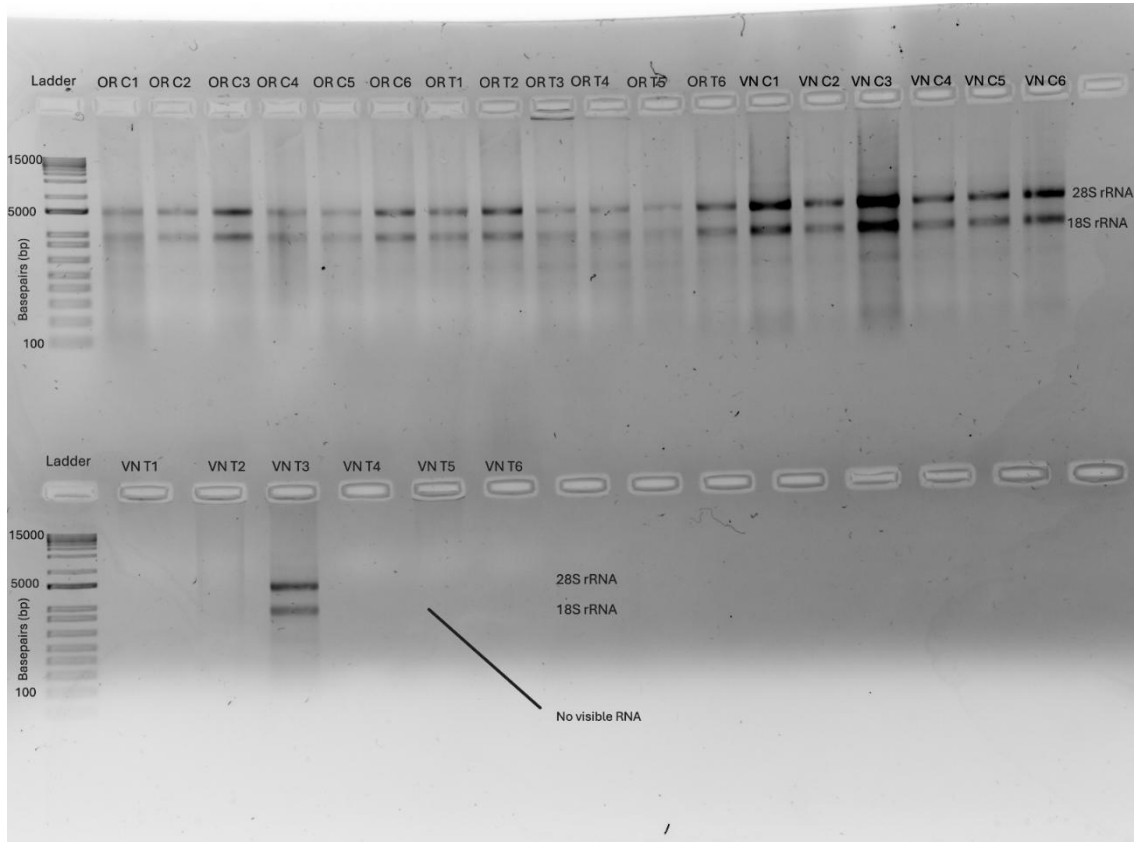


Figure 25 Batch 2, repeated RNA quality control for parental lines samples.

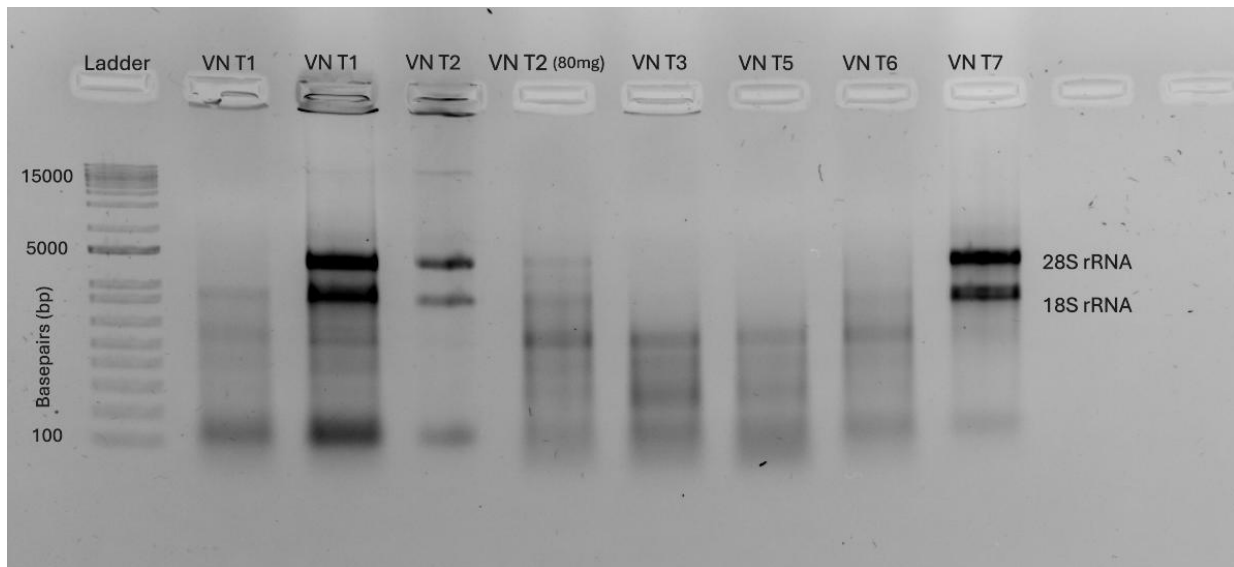


Figure 26: Repeated extraction for Vialone Nano treated samples in batch 2.

The repeated extraction of Vialone Nano treated samples in Batch 2 (Figure 27) similarly yielded 28S and 18S bands across all lanes except VN T3-T6, confirming successful RNA recovery. Bands differed in intensity across samples, consistent with the Qubit measured concentrations, but they

all showed 2:1 ratio indicating good integrity. All lanes displayed mild smearing below the 18S bands, this partial low weight smearing was observed consistently across this batch but did not preclude sample use, as the ribosomal bands remained intact and no DNA contamination was detected above 28S. VN T3 and VN T5 were degraded, due to a different extraction protocol which was used for their extraction.



Figure 27 Repeated extraction for Vialone Nano Control in Batch 2 (VN C6, VN C7, VNT3)

A final gel assessed three additional samples VN C6, C7 and VN T3. All the three samples showed the 28S and 18S rRNA bands, with darker bands intensity relative to earlier batches, consistent with higher RNA concentration. Smearing below the 18S band was present in all the lanes, however, the ribosomal bands remained intact and there was no DNA contamination observed. The samples were considered acceptable for downstream use.

#### 4.3.3. RNA Integrity Assessment by Agilent 2100 Bioanalyzer

RNA samples submitted to the IGA Technology Services facility (Udine, Italy) for RNA-seq library preparation, were analysed prior to library preparation, using Bioanalyzer, which provided the RNA Integrity Number (RIN) scores on a scale of 1-10, where values  $\geq 7$  are considered acceptable for RNA-seq. Samples identities for each well position are indicated in the figure captions below (Figure 28-30). Green highlighting denotes samples that met the RIN scores  $\geq 7$ .

### 4.3.3.1 Batch 1:IL273, IL189 ,Vialone Nano and *O.rufipogon*

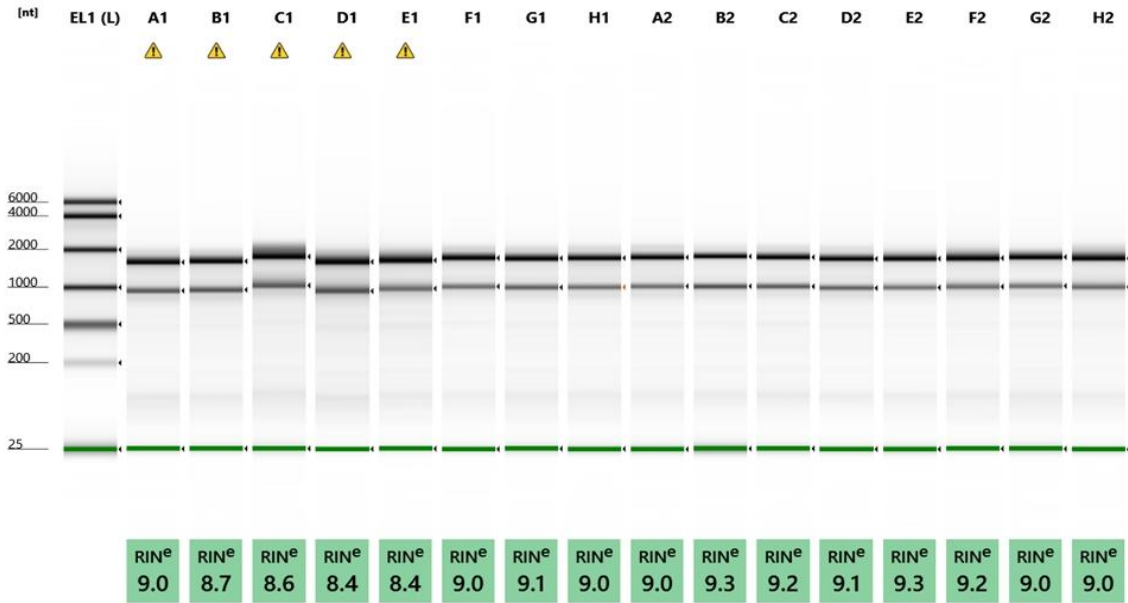


Figure 28: Bioanalyzer Digital Gel Image for samples in plate column 1-2. Lane order from left to right: A1=IL273C1, A2=IL189C3, B1=IL273C2, B2=IL189C5, C1=IL273C3, C2=IL189C6, D1=IL273C5, D2=IL189C7, E1=IL273C6, E2=IL273T1, F1=IL273C7, F2=IL273T2, G1=IL189C1, G2=IL273T3, H1=IL189C2, H2=IL273T5. Samples highlighted in green met the RIN  $\geq 7$  threshold and were selected for RNA-seq.

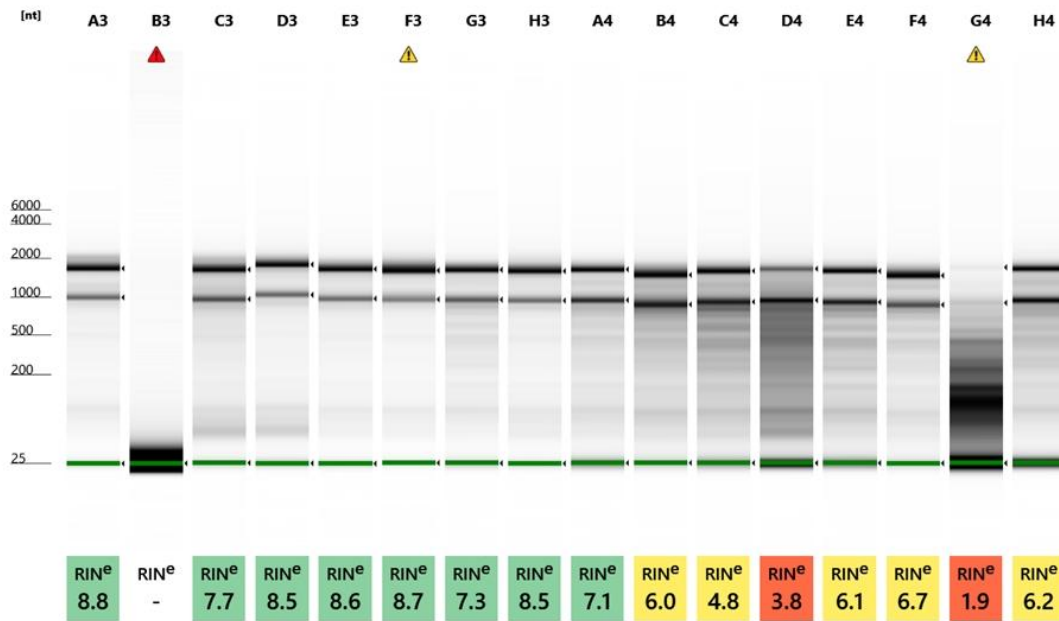


Figure 29: Bioanalyzer Digital Gel Image for samples in column 3-4 . Lane order from left to right: A3=IL273T6, A4=VNC1, B3=IL273T7, B4=VNC2, C3=IL189T1, C4=VNC3, D3=IL189T2, D4=VNC5, E3=IL189T3, E4=VNC6, F3=IL189T5, F4=VNC7, G3=IL189T6, G4=ORC1, H3=IL189T7, H4=ORC2. .Samples highlighted in green met the RIN  $\geq 7$  threshold and were selected for RNA-seq.

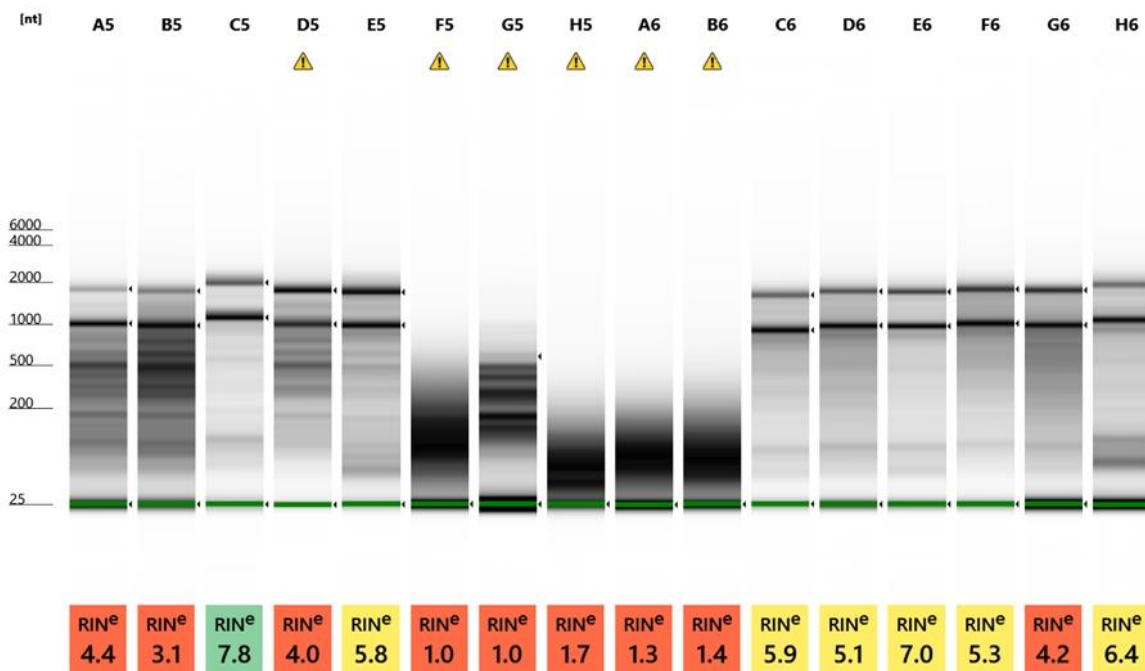
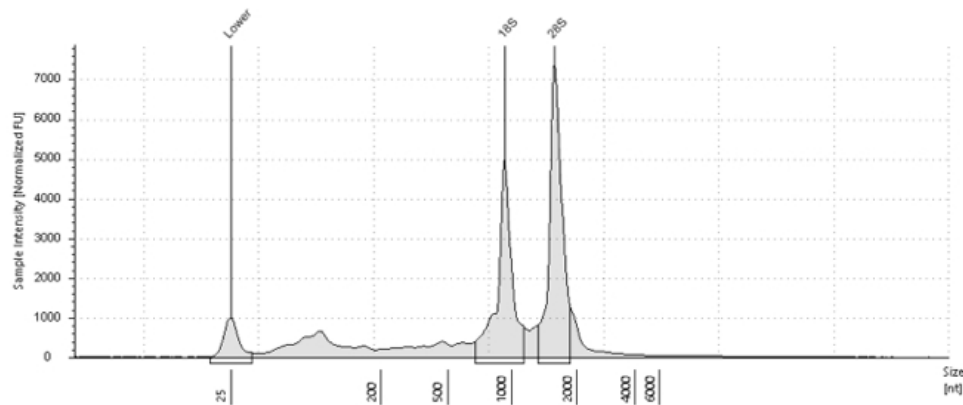


Figure 30: Bioanalyzer Digital Gel Image for samples in column 5-6 . Lane order from left to right: 5=ORC3, A6=VNT6, B5=ORC5, B6=VNT7, C5=ORC6, C6=ORT1, D5=ORC7, D6=ORT2, E5=VNT1, E6=ORT3, F5=VNT2, F6=ORT5, G5=VNT3, G6=ORT6, H5=VNT5, H6=ORT7. Samples highlighted in red were not selected for RNA-seq library preparation based on RIN scores  $\leq 7$ .

The initial Bioanalyzer gel images (Figure 28-30) provided an overview of RNA quality across all 48 samples. The electropherogram traces for individual samples were further analysed a Higher Sensitivity RNA ScreenTape system (Figure 31-34). Sample A1 (IL273 C1) showed high RNA integrity with a RIN score of 9.0, evidenced by sharp 18S and 28S ribosomal peaks and minimal baseline between them (Figure 31). Sample A2 (IL189 C3) produced a RIN score of 9.0, with sharp ribosomal bands confirming suitability for RNA-seq (Figure 32). These results are fully consistent with agarose gel data, where both IL273 and IL189 samples showed clear 28S and 18S bands throughout .

In contrast, sample H5 (VN T5) yielded a RIN score of 1.7, with absence of defined 18S and 28S peaks and extensive low molecular smearing indicating RNA degradation (Figure 33). Sample D5 (OR C7) showed a RIN score of 4.0, with an elevated baseline between the lower marker and ribosomal peaks consistent with moderate degradation (Figure 34). These results are consistent with the agarose gel electrophoresis data, where Vialone Nano treated samples and *O.rufipogon* samples from Batch 1 displayed comparable patterns of smearing and band loss.

A1: ID4152\_1-IL273C1-P1-A01-1:5



Sample Table

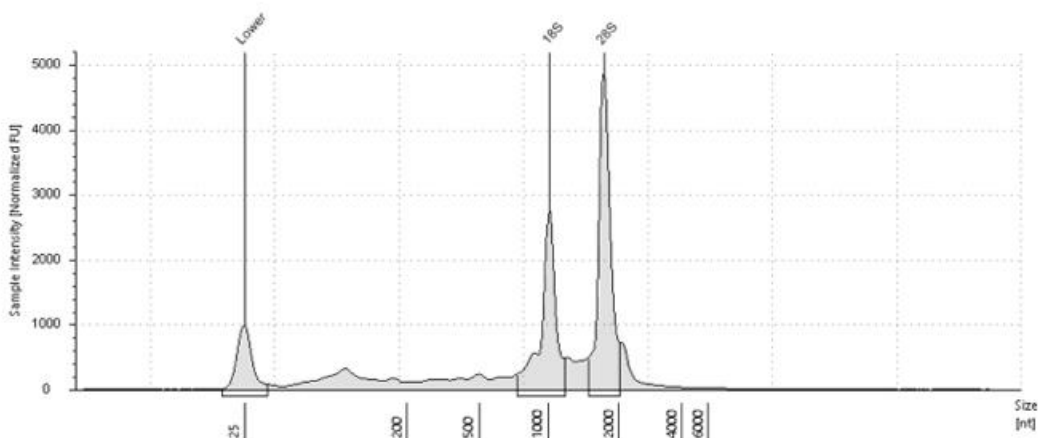
Well	RINe	28S/18S (Area)	Conc. [pg/ul]	Sample Description	Alert	Observations
A1	9.0	1.3	8330	ID4152_1-IL273C1-P1-A01-1:5	⚠	Caution! Expired ScreenTape device (used after two weeks of first use); RINe edited

Peak Table

Size [nt]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	700	700	82400	-		Lower Marker
933	2310	-	7290	42.65		18S edited
1579	3110	-	5790	57.35		28S edited

Figure 31: Sample A1(IL273 C1) RNA High Sensitivity Screen Tape

A2: ID4152\_9-IL189C3-P1-A02-1:5



Sample Table

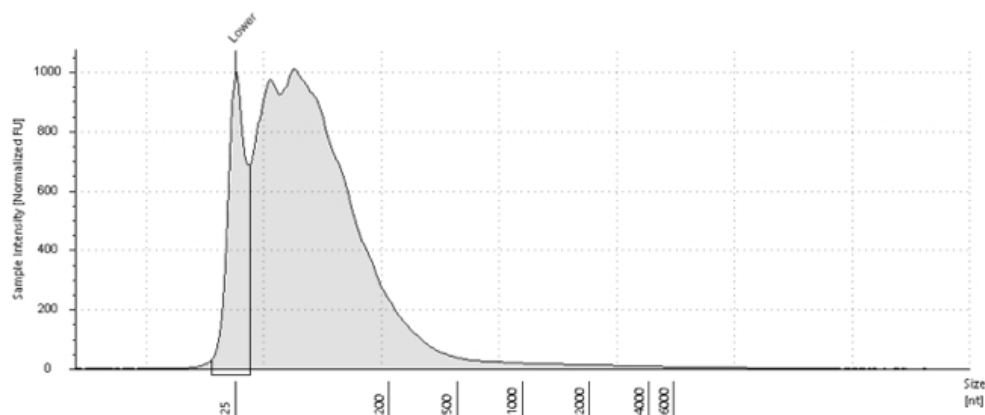
Well	RINe	28S/18S (Area)	Conc. [pg/ul]	Sample Description	Alert	Observations
A2	9.0	1.5	4700	ID4152_9-IL189C3-P1-A02-1:5		RINe edited

Peak Table

Size [nt]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	700	700	82400	-		Lower Marker
1018	1210	-	3490	40.64		18S edited
1730	1760	-	3000	59.36		28S edited

Figure 32: Sample A2 (IL189 C3) RNA High Sensitivity Screen Tape

H5: ID4152\_40-VNT5-P1-H05-1:5



Sample Table

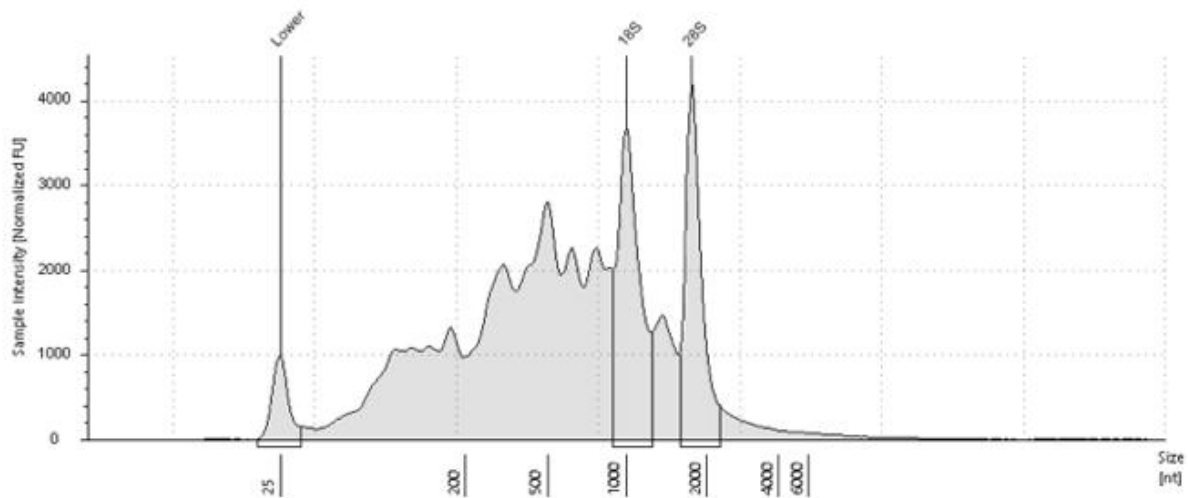
Well	RINe	28S/18S (Area)	Conc. [pg/ul]	Sample Description	Alert	Observations
H5	1.7	-	2520	ID4152_40-VNT5-P1-H05-1:5	⚠	The lower ribosomal fragment is missing

Peak Table

Size [nt]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	700	700	82400	-		Lower Marker

Figure 33: Sample H5 (VN T5) RNA High Sensitivity Screen Tape

D5: ID4152\_36-ORC7-P1-D05-1:5



Sample Table

Well	RINe	28S/18S (Area)	Conc. [pg/ul]	Sample Description	Alert	Observations
D5	4.0	0.8	15600	ID4152_36-ORC7-P1-D05-1:5	⚠	Sample concentration outside recommended range; RINe edited

Peak Table

Size [nt]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
25	700	700	82400	-		Lower Marker
1007	2330	-	6800	54.65		18S edited
1762	1930	-	3220	45.35		28S edited

Figure 34: Sample D5 (ORC7) RNA High Sensitivity Screen Tape

The Bioanalyzer results confirmed and quantified the patterns already identified by agarose gel electrophoresis: IL273 and IL189 samples consistently produced high RIN scores ( $\geq 7$ ), while parental lines samples Vialone Nano and selected *O.rufipogon* from Batch 1 showed lower RIN values below the acceptable threshold for RNA-seq. The low RIN score observed in H5 (VN T5, RIN 1.7) and D5 (OR C7, RIN 4.0) alongside other parental line samples shows why repetition of the water stress experiment and RNA extraction was necessary for the parental lines. Bioanalyzer assessment of Batch 2 confirmed that the majority of samples met the  $RIN \geq 7$  threshold required for RNA-seq library preparation, with several samples achieving RIN values above 9.0. Samples failing to meet this threshold were excluded from sequencing. RNA sequencing is currently underway at w IGA Technology Service (Udine, Italy) ; differential gene expression analyses will be conducted upon receipt of the sequencing data.

4.3.3.2 Batch 2 Vialone Nano and *O.rufipogon* Repeated Extraction

The results from the second batch showed a notable improvement overall, with the majority of samples yielding higher RIN values compared to batch 1. Samples identities for each well position are indicated in the figure captions below (Figure 33-35).

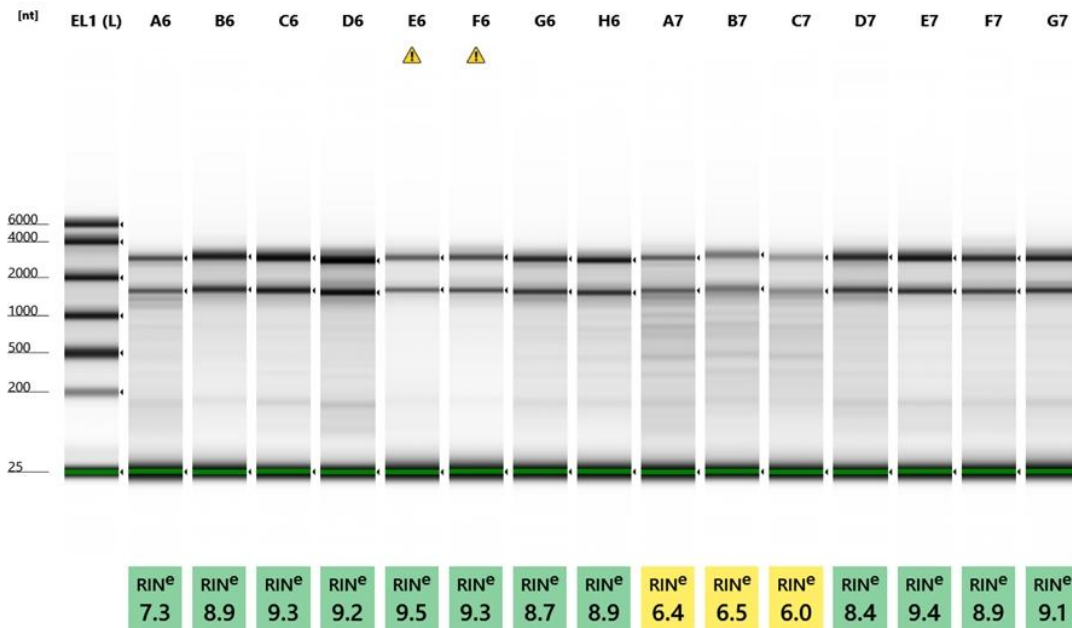


Figure 35 Bioanalyzer Digital Gel Image for Batch 2 samples in plates from left to right: ORC1, ORT3, ORC2, ORT4, ORC3, ORT5, ORC4, ORT6, ORC5, VNC1, ORC6, VNC2, ORT1, VNC3, ORT2, VNC4. Samples highlighted in green met the RIN  $\geq 7$  threshold and were selected for RNA-seq.

Figure 35 displays the gel image for well A6 to G7, corresponding to samples ORC1-ORC6, ORT1-ORT6, and VNC1-VNC3. The RNA ScreenTape image shows slight smearing in several samples, which is more pronounced in wells A7, B7 and C7, corresponding to ORT3, ORT4 and ORT5- the *O. rufipogon* treated samples with RIN values below 7 (6.4, 6.5, and 6.9 respectively). Among the *O. rufipogon* samples, E6 (ORC5) recorded the highest RIN of 9; however, its concentration fell below the functional value at 18ng/ $\mu$ L. The overall improvement across samples suggests that consistent use of liquid nitrogen at every step of extraction, combined with careful RNA handling, is critical for obtaining high quality RNA. Figure 35 presents the concentration and RIN values for all samples in this batch.

Vialone Nano samples also showed considerable improvement relative to Batch 1, though five samples still recorded RIN values below 7. The control samples (well E7, F7 and G7, as well as A8 and B8, corresponding to VNC1-VNC6) all achieved good RIN values, with the highest

reaching 9.4, a value shared by three treated samples. For the inoculated Vialone Nano samples, material collected in April 2025 was designated for sequencing. Trial samples F8 and G8 (VNT5 and VNT6) were run to assess RNA quality; these recorded RIN values of 6.5 and 6.1, respectively, indicating they were not suitable for sequencing.

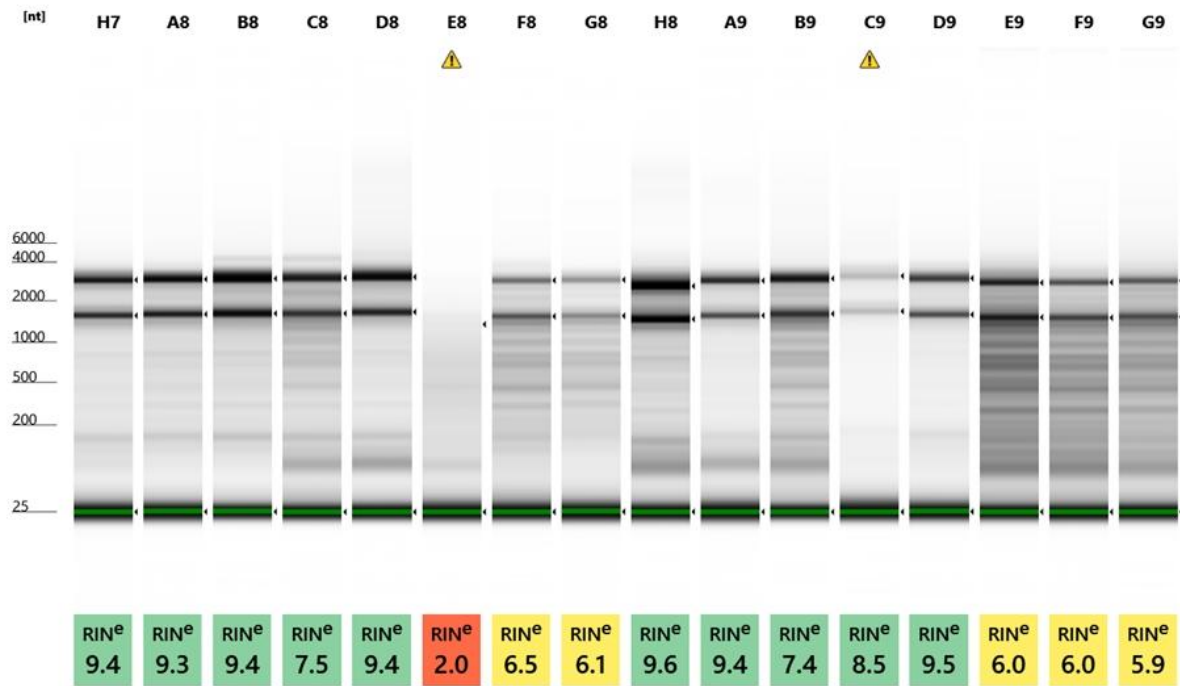


Figure 36 Bioanalyzer Digital Gel Image for Batch 2 samples .Lane order from left to right: VNC5, VN101, VNC6, VN102, VNT1, VN103, VNT2, VN105, VN106, VNT5, VN107, VNT7, VN203, IL273T7. Note E8 were empty. Samples highlighted in green met the RIN  $\geq 7$  threshold and were selected for RNA-seq.

The treated samples designated for sequencing (wells A9-G9, corresponding to VN101-VN107 and VN203) showed variable quality D9(VN105) achieved the highest RIN of 9.5, while E9, F9 and G9 had the lowest values at 6.0, 6.0 and 5.9, respectively. Figure summaries the concertation and RIN values for all samples, and Figure 36 shows the RNA ScreenTape gel with corresponding RIN values displayed beneath each lane .

Representative electropherogram generated by the Agilent 2100 Bioanalyzer are shown in Figure 37-39, illustrating the ribosomal RNA peaks and baseline regions used to calculate RIN values. Figure 37 shows the electropherogram for *O.rufipogon* sample ORC3 (RIN 9.3), which recorded the highest RIN among the *O.rufipogon* samples in this batch. Figure 38 shows Electropherogram

for Vialone Nano samples VNC4 (well H7, RIN 9.4), representing one of the highest-quality samples in the batch. In contrast, Figure 39 shows the electropherogram for samples VN203 (well G9, RIN 5,9), one of the lowest-quality samples, where degradation is reflected in a less defined ribosomal peak profile.

C6: ID4152\_3-ORC3-P1-C01



Sample Table

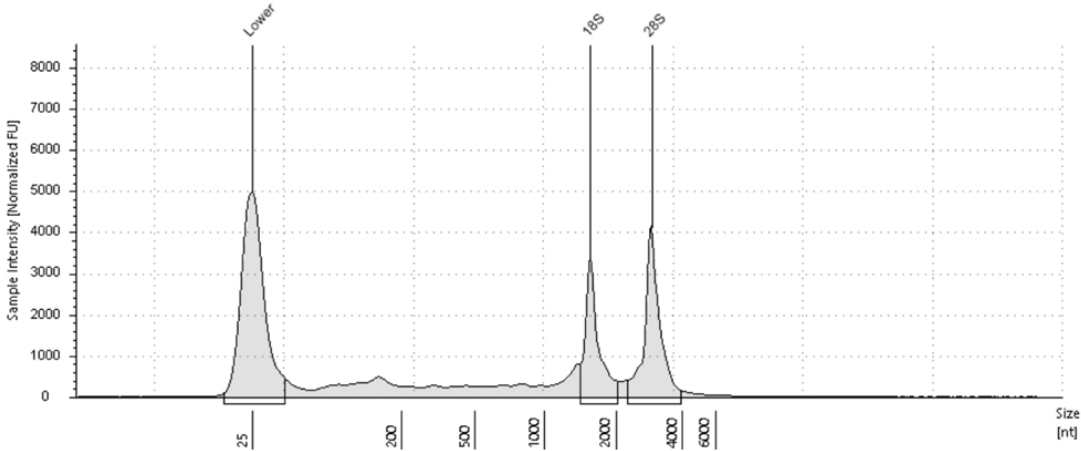
Well	RINe	28S/18S (Area)	Conc. [ng/ul]	Sample Description	Alert	Observations
C6	9.3	1.1	46.7	ID4152_3-ORC3-P1-C01		

Peak Table

Size [nt]	Calibrated Conc. [ng/ul]	Assigned Conc. [ng/ul]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
25	36.0	36.0	4240	-		Lower Marker
1576	15.0	-	28.1	47.85		18S
2916	16.4	-	16.5	52.15		28S

Figure 37: Electropherogram of *O. rufipogon* control sample ORC3 generated by the Agilent 2100 Bioanalyzer, showing well-defined 18S and 28S ribosomal RNA peaks indicative of high RNA integrity (RIN=9.3)

H7: ID4152\_16-VNC4-P1-H02

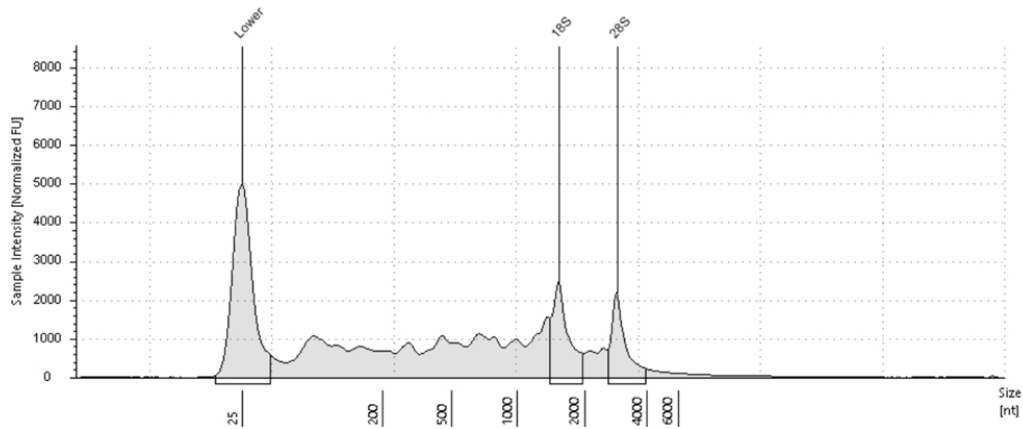


Sample Table

Well	RINe	28S/18S (Area)	Conc. [ng/ul]	Sample Description	Alert	Observations
H7	9.4	1.4	37.2	ID4152_16-VNC4-P1-H02		

Figure 38 Electropherogram of Vialone Nano treated samples VNC4 (well H7) generated by the Agilent 2100 Bioanalyzer, showing high RNA integrity (RIN=9.4) as indicated by the diminished and broadened ribosomal RNA peak profile

G9: ID4152\_31-VN203-P8-G04



Sample Table

Well	RINe	28S/18S (Area)	Conc. [ng/ul]	Sample Description	Alert	Observations
G9	5.9	0.8	65.4	ID4152_31-VN203-P8-G04		

Figure 39 Electropherogram of Vialone Nano treated samples VN203 (well G9) generated by the Agilent 2100 Bioanalyzer, showing reduced RNA integrity (RIN 5.9) as indicated by the diminished and broadened ribosomal RNA peak profile.

Sample Info

Well	RINe	28S/18S (Area)	Conc. [ng/ul]	Sample Description	Alert	Observations
EL1	-	-	70.4	Electronic Ladder		Ladder
A6	7.3	1.2	32.2	ID4152_1-ORC1-P1-A01		
B6	8.9	1.1	40.0	ID4152_2-ORC2-P1-B01		
C6	9.3	1.1	46.7	ID4152_3-ORC3-P1-C01		
D6	9.2	1.4	62.5	ID4152_4-ORC4-P1-D01		
E6	9.5	1.1	18.4	ID4152_5-ORC5-P1-E01	⚠	Sample concentration outside functional range for RINe and the assay
F6	9.3	1.1	20.4	ID4152_6-ORC6-P1-F01	⚠	Sample concentration outside functional range for RINe and the assay
G6	8.7	1.1	36.9	ID4152_7-ORT1-P1-G01		
H6	8.9	1.0	36.4	ID4152_8-ORT2-P1-H01		
A7	6.4	0.8	40.7	ID4152_9-ORT3-P1-A02		
B7	6.5	0.8	38.9	ID4152_10-ORT4-P1-B02		
C7	6.0	0.7	31.8	ID4152_11-ORT5-P1-C02		
D7	8.4	1.2	46.0	ID4152_12-ORT6-P1-D02		
E7	9.4	1.2	38.2	ID4152_13-VNC1-P1-E02		
F7	8.9	1.4	35.9	ID4152_14-VNC2-P1-F02		
G7	9.1	1.4	36.6	ID4152_15-VNC3-P1-G02		
H7	9.4	1.4	37.2	ID4152_16-VNC4-P1-H02		
A8	9.3	1.5	43.0	ID4152_17-VNC5-P1-A03		
B8	9.4	1.2	63.8	ID4152_18-VNC6-P1-B03		
C8	7.5	1.0	59.8	ID4152_19-VNT1-P1-C03		
D8	9.4	1.3	57.1	ID4152_20-VNT2-P1-D03		
E8	2.0	-	18.0	ID4152_21-VNT3-P1-E03	⚠	Sample concentration outside functional range for RINe and the assay; The upper ribosomal fragment has degraded
F8	6.5	0.7	41.2	ID4152_22-VNT5-P1-F03		
G8	6.1	1.0	28.5	ID4152_23-VNT7-P1-G03		
H8	9.6	1.5	76.2	ID4152_24-IL273T7-P1-H03		
A9	9.4	1.5	31.0	ID4152_25-VN101-P2-A04		
B9	7.4	1.0	70.1	ID4152_26-VN102-P3-B04		
C9	8.5	1.1	12.1	ID4152_27-VN103-P4-C04	⚠	Sample concentration outside functional range for RINe and the assay
D9	9.5	1.1	26.5	ID4152_28-VN105-P5-D04		
E9	6.0	0.7	103	ID4152_29-VN106-P6-E04		
F9	6.0	0.7	71.5	ID4152_30-VN107-P7-F04		
G9	5.9	0.8	65.4	ID4152_31-VN203-P8-G04		

Figure 40 Concentration and RIN values for all Batch 2 samples

Overall, the Batch 2 results represent a clear improvement over Batch 1, attributable to more consistent RNA handling practises and the rigorous use of liquid nitrogen through the extraction workflow. All samples with  $RIN \geq 7$  from both batches were confirmed suitable and submitted for RNA sequencing at IGA Technology Services (Udine, Italy).

## DISCUSSION

This study was aimed to: (i) map the colonisation patterns of *K. sacchari* across a diverse introgression panel and its parental lines (Vialone Nano and *O.rufipogon* ); (ii) evaluate whether *Kosakonia sacchari* inoculation improves water stress, enhances water stress tolerance and growth parameters such as relative water content, shoot length; and (iii) determine how host gene expression varies between 4 genotypes in response to bacterial colonisation following water stress.

### 5.1 Differential Root Association of *K. sacchari* Colonisation within Rice Introgression Panel

Root colonisation by *Kosakonia sacchari* revealed significant differences across 21 introgression lines and parental varieties suggesting that colonisation efficiency is genotype-dependent. *Oryza rufipogon* had the highest colonisation (8.23 log<sub>10</sub>CFU/g) and Vialone Nano had the lowest colonisation among the parental lines (7.29 log<sub>10</sub>CFU/g), while the mean CFU for the introgression lines ranged from 6.07 to 8.17 log<sub>10</sub>CFU/g. IL8 and IL6 showed significantly lower colonisation, with achieving 6.07 log<sub>10</sub>CFU/g and IL6 achieving 6.46 log<sub>10</sub>CFU/g values below lowest parental line recorded for Vialone Nano (7.29 log<sub>10</sub>CFU/g), indicating a wide quantitative range in the introgressed population. These results confirm H1 hypothesis, since several introgression lines significantly differed from the recipient parents in the CFU levels and H2 hypothesis, because the wild donor supported higher endophytic colonisation than the elite cultivars.

*O. rufipogon* had the highest CFU levels (8.23 log<sub>10</sub>CFU/g  $\approx$  8.28  $\times$  10<sup>8</sup> CFU/g) across all varieties, higher than values reported by (Koomnok et al., 2007) who found 5.25 $\times$ 10<sup>6</sup> g<sup>-1</sup> in fresh roots using most probable number method in N-free semisolid media. However, these CFU level estimates are not directly comparable two reasons. Koomnok et al. (2007) did not use *K.sacchari* RCA25, they quantified native diazotrophic endophytes belonging to the genera *Azospirillum*, *Herbaspirillum*, *Beijerinckia* and *Pseudomonas*, which are entirely different bacterial species with distinct colonisation biology. Second, their MPN method in N-free media selectively detects bacteria capable of growing as microaerophilic nitrogen fixation, whereas our CFU values reflect populations of an actively inoculated *K. sacchari* strain recovered on Lysogeny Broth (LB) medium supplemented with vancomycin, which strongly favours *K. sacchari* growth. The CFU values observed in this study should therefore be interpreted as evidence that *O. rufipogon* can

sustain dense populations of an introduced diazotrophic strain, rather than as a contradiction of previous estimates of diazotrophic bacteria in wild rice .

### **5.1.1 High colonisation capacity of *O. rufipogon***

The differences in CFU levels across the introgression panel and the parental lines support the view that endophytic association is a quantitative trait, in which alleles derived from *O. rufipogon* can either restore or fail to restore the wild-like colonisation capacity in the Vialone Nano genetic background. Continuous quantitative variation in plants-microbe traits has been described within rice. Studies comparing wild *Oryza* species and cultivated *O. sativa* show that different rice genotypes assemble distinct endophytic communities, with wild relative such as *O. officinalis* and *O. rufipogon* harbouring higher endophytic diversity and stronger enrichment of plant-growth promoting taxa than cultivated varieties. These patterns indicate that multiple rice loci, rather than a single gene, control microbiome composition and abundance, consistent with the QTL-like behaviour observed in the introgression panel (Lei et al., 2025; Tian et al., 2023).

The high colonisation levels seen in *O. rufipogon* can be explained by several mechanism, including root architecture, exudate composition, and immune regulation. Studies have shown that enrichment of nitrogen fixing, Indole-3-acetic acid (IAA) producing and 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) positive taxa such as *Enterobacter*, *Bacillus*, *Pseudomonas* and *Kosakonia* represents some of the plant growth-promoting functions demonstrated in wild *Oryza* relatives including, *O. rufipogon* and *O. officinalis* which assemble root and endophytic communities with higher diversity. This suggests that wild genomes encode exudate profiles and immune regulatory settings that favour stable internal colonisation rather than exclusion of endophytes (Koomnok et al., 2007; Tian et al., 2023).

Wild roots secrete richer blends of sugars, amino acids, and organic acids that serve as chemoattractants and carbon source for diazotrophic endophytes (Chaparro et al., 2013; Pérez-Jaramillo et al., 2016). Studies from *O. rufipogon* and other wild *Oryza* species show that rhizosphere and endosphere communities are strongly linked to metabolic pathways for carbon and nitrogen acquisition; specifically, exudates such as citrate and malate correlate with a higher abundance of nitrogen-fixing genera such as *Herbaspirillum* and *Azospirillum* (Lei et al., 2025; Tian et al., 2023). Such exudation patterns provide an ideal niche for strains like *K. sacchari*, which require readily available carbon and signalling molecules to initiate attachment and penetration.

This is consistent with the broad metabolic versatility of *K. sacchari*; as previously noted (Chen et al., 2014b), this species efficiently utilises a diverse array of sugars and amino acids-including D-fructose, D-mannitol, and L-alanine which are prevalent in the rich exudate profile of wild *Oryza* species

Wild rice also appears to maintain a more permissive yet controlled immune environment for endophytes. Endophytic diazotrophs isolated from *O. rufipogon* frequently express cell wall degrading enzymes such as pectinases and cellulases at levels sufficient for entry but not enough to trigger strong immune responses(Hardoim et al., 2015; Tian et al., 2023). Wild *Oryza* genotypes often exhibit extensive root systems with higher lateral root density and root hair proliferation than japonica cultivars, increasing the number of natural colonisation sites where endophytes typically enter particularly at lateral root emergence zones and root hair bases(Lei et al., 2025).The differences in root microbiota composition between *O.rufipogon* and *O.sativa* further confirm that wild rice genotype and phenotype exert a strong influence on recruiting and retaining specific microorganism, a capacity that has been selectively reduced during domestication (Chang et al., 2025b; Jiang et al., 2024).

### **5.1.2 Reduction in endophyte colonisation in Vialone Nano**

The significantly lower colonisation efficiency of *K. sacchari* observed in Vialone Nano relative to *O.rufipogon* (7.29 log<sub>10</sub>CFU/g) suggests that the transition from wild to domesticated rice has substantially altered the capacity of Vialone Nano to sustain productive endophyte associations. This is consistent with the broader literature documenting a systemic erosion of plant-microbe interaction traits during crop domestication, where artificial selection for agronomic yields targeted grain size, tillering architecture, and other traits, inadvertently disrupting the host's capacity to recruit and maintain beneficial microbiota (Bulgarelli et al., 2015; Pérez-Jaramillo et al., 2016).

Soldan et al. (2021) proposed the 'double leash' model to explain this erosion. In wild plant population, natural selection continuously reinforces the plant's ability to shape and manage its own microbiome. This represents the first level of control or 'leash,' that the host plant maintains over its microbial community. Domestication, however, introduced the second layer of selection pressure in which breeders and farmers selected for traits that improved crop yield and stability, rather than traits that optimise plant-microbe interactions. Since the phenotypic changes introduced

by artificial selection for traits such as larger seeds, modified root architecture, and reduced metabolite production, were never evaluated for their impact on microbial recruitment, they have gradually weakened the host-to -microbe assembly capacity. As a result, domesticated cultivar such as Vialone Nano can still retain a genetic capacity to be colonised, but they have lost the precise molecular and biochemical signals that promote deep and sustained endophytic association with bacteria such as *K. sacchari*.

The reduced colonisation efficiency observed in Vialone Nano in the present study is not an isolated finding. Andreozzi et al. (2019) reported similar results when two *O.sativa* cultivars, Baldo and Vialone Nano were inoculated with diazotrophic endophytes *Enterobacter cloacae* RCA25 and *Herbaspirillum hettiense* RCA24. Andreozzi et al. (2019) used the same bacteria isolate, at the time classified as *Enterobacter cloacae* RCA25; subsequent taxonomic revision reclassified this strain as *Kosakonia sacchari* RCA25, and it is referred to by its current designation through this thesis.

Under identical inoculation conditions, Baldo achieved root colonisation counts of approximately 13-fold higher than Vialone Nano from the same strain RCA25. The physiological results followed a similar pattern: Baldo plants received co-inoculation showed significant improvement in chlorophyll content, shoot dry weight (up to 89%) and shoot nitrogen content (up to 80%), while Vialone Nano showed no measurable benefit. (Andreozzi et al., 2019) concluded that host specificity can exist among endophytic bacteria, implying that Vialone Nano has reduced capacity for endophytic associations with bacteria such *K. sacchari*.

Several biological mechanisms may explain why Vialone Nano has reduced affinity, most notably root exudates compositions. Root exudates composition is a primary determinant of endophytic recruitment. Domestication has been shown to alter the quantity and quality of root secreted exudates in a way that reduce selective attraction of beneficial diazotrophs (Chaparro et al., 2013; Pérez-Jaramillo et al., 2016). Japonica rice cultivar such as Vialone Nano are characterised by reduced secondary metabolites production, enlarged seed and grain morphology, and altered root architecture. Seed size enlargement may affect the initial stages of microbial community assembly, as larger seeds absorb water more slowly during germination, changing the exudates released into the surrounding soil and can shift the microbial community established around the seedlings at the earliest stages of growth, specifically the community from which early root-colonising

endophytes are recruited. These changes suggest that Vialone Nano has progressively moved away from the selective host-to-microbe control mechanism that defined the *O.rufipogon* wild ancestor's relationship with its endophytic community(Soldan et al., 2021).

### **5.1.3 Introgression Line Variation in colonisation affinity**

The introgression line panel revealed significant variation in *K. sacchari* colonisation efficiency. A number of lines achieved colonisation levels comparable to those of the wild parent, *O.rufipogon*, while others showed colonisation patterns resembling the low colonisation efficiency of Vialone nano, with CFU values ranging from 6.07 to 8.17 log<sub>10</sub>CFU/g. This variation in CFU levels suggests that chromosomal segments from *O.rufipogon* carry specific loci that have a substantial and disproportionate effect on host-endophyte compatibility. This pattern is similar to what has been observed in other studies where diseases resistance traits were successfully recovered through introgression lines from wild relatives (Arora et al., 2019). In common beans, differences in rhizosphere microbiome composition of wild and domesticated cultivars were similarly attributed to specific genomic regions linked to root architecture and exudates chemistry (Pérez-Jaramillo et al., 2016).

The dispersion of colonisation phenotypes across the IL panel is consistent with colonisation affinity being a quantitative trait controlled by multiple loci rather than a single gene. The wide CFU range observed across the panel reflects the differential introgression of *O.rufipogon* genomic segments that individually or collectively contribute to host-endophyte compatibility. ILs carrying introgressions at chromosomal regions that harbour favourable alleles for example, those influencing root exudates chemistry, cell wall composition, or immune signalling would be expected to show elevated colonisation, while ILs lacking those segments would revert towards the Vialone Nano phenotype. This is similar to the architecture of other quantitative stress-tolerance traits in rice, where QTL mapping studies have repeatedly identified multiple genomic intervals of moderate-to-large effect contributing to the overall phenotype (Jia et al., 2019; Vikram et al., 2011).

The IL variation documented in the present study provides proof of concept that domestication-associated reductions in endophyte affinity are reversible. Targeted chromosomal introgression from *O.rufipogon* was sufficient to partially reconstitute the colonisation phenotype in the domesticated background, confirming the relevant host-side loci remain functionally active in the

wild parent genome and are not inherently incompatible with the Vialone Nano genetic architecture. *O.rufipogon* seeds harbour an exceptionally rich endophytic community, in which approximately 96 % of cultivable isolates produce indole-3 acetic acid (IAA), nearly 60% carry N-fixation capacity and over 78% can solubilize phosphate- a multi-trait functional profile that Z. Zhang et al. (2021) demonstrated was directly transferable to cultivated rice hosts, significantly improving shoot length, root length and dry weight, and chlorophyll content upon inoculation. Introgression lines that recover *O.rufipogon* level colonisation affinity would therefore be better positioned to benefit from bioinoculant applications.

The colonisation phenotypes documented across the 41ILs in the present study, combined with the DNA extracted from drought stress test, provide a dataset for future quantitative trait locus (QTL) mapping aimed at identifying the specific *O.rufipogon* -derived genomics responsible for high colonisation affinity. This work is not limited to 41 lines characterised here; the genetic laboratory currently holds a broader population of nearly 200 phenotyped ILs, and integrating colonisation data across this panel will increase the statistical power available for QTL detection and candidate gene identification.

## **5.2 Relative Water Content (RWC) Under Stress**

Relative water content (RWC) was used as the indicator for cellular hydration status under the abiotic stress conditions in this experiment. RWC is considered a reliable parameter to measure water deficit in leaves, as it captures the net balance between water uptake from the roots and transpirational water loss through the leaves. RWC reflects the hydration state of the mesophyll cells and is therefore closely linked to the maintenance of enzyme activity, stomatal function and carbon assimilation capacity. Under abiotic stress such as drought or salinity, a decline in RWC represents early signs of cellular water imbalance, followed by stomatal closure, impaired photosynthesis, accumulation of reactive oxygen species (ROS), and increased membrane lipid peroxidation, which is measurable as malondialdehyde (MDA) (Abdelaal et al., 2021).

The inoculation of rice genotypes with *Kosakonia sacchari* was expected to exert a measurable protective effect on RWC under stress, based on the theory that plant growth-promoting bacteria improve plant water relations through a combination of mechanisms, including enhanced root architecture through indole-3 acetic acid (IAA) production, exopolysaccharides (EPS)-mediated water retention, and hormonal modulation of stomatal aperture. *Herbaspirillum seropedicae* and

*Azospirillum brasilense*, which are also Gram negative diazotrophic endophytes in graminaceous crops have both shown to significantly maintain RWC and membrane stability in inoculated wheat and maize plants under water stress.

Introgression lines derived from *O.rufipogon* have been shown to demonstrate enhanced drought tolerance capacity; Qin et al. (2019) reported that ILs overexpressing multi-effect locus *NALI* (Narrow leaf 1), a gene encoding a protein involved in regulating leaf development, acts as multi-trait QTL influencing leaf width, chlorophyll content and plant architecture (G.-H. Zhang et al., 2014), with overexpression associated with significantly higher RWC and lower malondialdehyde (MDA) than controls under simulated drought (Qin et al., 2019). This suggests that *O.rufipogon* introgressions in the genetic background of ILs used in this study may predispose them to superior water retention, independent of bacterial inoculation (Abdelaal et al., 2021).

Contrary to these expectations, the results did not reveal statistically significant differences in RWC between treated and non-treated plants across the four genotypes (IL189, IL273, *O.rufipogon* and Vialone nano). One explanation for this may lie in the functional profile of *Kosakonia sacchari* relative to other well-studied plant growth-promoting bacteria. The genome of *K. sacchari* type strain SP1T is primarily characterised by genes associated with nitrogen fixation, carbohydrate transport, and amino acid metabolism (Chen et al., 2014b). Unlike *Pseudomonas* and *Bacillus* species, which are well known for the ACC deaminase activity, an enzyme that cleaves the ethylene precursor ACC, thereby reducing stress-induced ethylene accumulation and maintaining turgor pressure and RWC under drought (Glick, 2012; Saikia et al., 2018) published *K. sacchari* SP1T genome characterisation does not identify ACC deaminase as a core functional trait (Chen et al., 2014b). While deaminase activity has been reported in some *Kosakonia* isolates (Romano et al., 2020), strain RCA25 has not been specifically characterised for this activity, and its primary growth-promoting function remains biological nitrogen fixation. This strain-specific functional limitation may therefore partly account for the absence of a significant inoculation effect on RWC under the stress conditions applied in this study.

Chen et al. (2014b) reported that nitrogen fixation mechanism of *K. sacchari* is a highly oxygen and energy sensitive biochemical process. Nitrogenase activity in diazotrophic endophytes is one of the functions to be suppressed under abiotic stress conditions, particularly osmotic stress and salinity, which disrupt the energetic and redox environment required for the *nif* gene system

(Bueno Batista and Dixon, 2019; Madkour et al., 1990). The nitrogen-fixing capacity of *K. sacchari* may therefore have been inactivated by the stress conditions applied in this experiment. Importantly, the presence of viable *K. sacchari* colonies across all plated samples confirms that the bacterium was not eliminated from the root endosphere under stress conditions. Rather, the absence of a growth-promoting effect is more consistent with metabolic suppression than with colonisation failure. Under osmotic stress, diazotrophic endophytes are known to divert energy towards osmoprotectant synthesis for self-preservation directly competing with the energy demands of nitrogenase activity (Chen et al., 2014b; Madkour et al., 1990). Drought induced changes in the roots apoplast including ROS accumulation and apoplastic acidification further create a redox environment incompatible with nitrogenase function (Gil-Quintana et al., 2013; Naya et al., 2007). The bacteria were therefore likely present in a viable but growth promoting-inactive state, physically colonising the root tissue while contributing negligible fixed nitrogen or IAA to the host during the stress period. Measuring foliar nitrogen content or deploying  $^{15}\text{N}$  isotope dilution in future stress experiments would provide more direct evidence of whether *K. sacchari* nitrogen fixation remains active under stress and would serve as a more sensible indicator of bacterial functional activity than RWC alone (Boddey et al., 1995).

The results of IL273 were striking and unexpected. IL273 had the lowest CFU levels among the four genotypes and was selected for its contrasting colonisation characteristics. It was therefore expected to show the lowest overall RWC. Instead IL189 performed well comparably to the other genotypes despite its poor colonisation efficiency. This may be explained by abiotic stress tolerance capacity conferred by *O. rufipogon* derived introgressions present in IL189's background (Wang et al., 2017b; Zhou et al., 2006). Introgression lines carrying wild rice genomic segments have been shown to harbour alleles for stress-relevant traits, including QTLs for drought and salt tolerance, that can operate independently of microbial associations (Wang et al., 2017b; Zhang et al., 2022; Zhou et al., 2006). The genotypic differences in RWC observed across all the four lines suggest that, under conditions tested, RWC was primarily determined by intrinsic water-use efficiency for each genotype, with bacterial inoculation contributing insufficient additional stress relief. This interpretation is consistent with reports that PGPB effects on water-related parameters tend to be more pronounced under severe and prolonged stress conditions and may be masked under moderate stress levels and short experimental periods (Abdelaal et al., 2021; Batool et al., 2020; Giri et al., 2025).

Taken together, the absence of significant inoculation effect on RWC is better explained by strain-specific functional limitations and stress-induced metabolic suppression than colonisation failure. *K. sacchari* lacks the ACC deaminase activity that is central to ethylene-mediated stress relief in better characterised PGPB species, and its primary growth promoting mechanism-biological nitrogen fixation is itself suppressed by the osmotic and redox disruptions imposed by drought stress (Glick, 2012; Madkour et al., 1990; Naya et al., 2007; Saikia et al., 2018). Future experiments incorporating foliar nitrogen content or <sup>15</sup>N isotope dilution assays would provide more direct evidence for whether *K. sacchari* nitrogen fixation remains active under stress conditions and would allow a clear separation of bacterial functional contributions from the intrinsic drought tolerance capacity of the host genotype (Boddey et al., 1995).

### 5.3 Shoot Length Analysis

Shoot length revealed clear differences between genotypes depending on inoculation. IL273 and IL189, non-inoculated control plants achieved greater shoot length than inoculated plants under both 100% and 50% field capacity conditions, suggesting that *K. sacchari* inoculation conferred no shoot growth benefits and was in some cases associated with reduced elongation under stress. One possible explanation for reduced elongation in inoculated plants is the metabolic cost imposed on the host by establishing and maintaining an endophytic association. Accommodating an endophyte within root tissue requires the plants to invest carbon and energy in membrane remodelling, immune suppression and apoplastic niche formation- resources that would be otherwise directed toward shoot growth (Hardoim et al., 2015). Vialone Nano followed the same trends, uninoculated controls consistently outperformed the inoculated plants but exhibited a distinct response pattern to other genotypes. Inoculate Vialone Nano showed a paradoxical inverse in response to water availability, achieving short shoot under well-water conditions (6.43cm at 100% FC) than under drought stress (8.67cm at 50% FC). This illustrates that *K. sacchari* RCA25 interacts with host genotypes in a highly genotype-specific manner, consistent with significant Genotype × Treatment interaction detected by the GLM ( $p= 0.007$ ). This genotype × inoculation interaction is consistent with the broader PGPB literature, which recognises that bacterial growth-promotion effects are not universal but highly dependent on host genotype, soil conditions and the specific stress regime applied (Andreozzi et al., 2019; Etesami, 2025; Glick, 2012).

The absence of an inoculation-mediated shoot length increase in all genotypes IL273, IL189, Vialone Nano and *O.rufipogon* can be interpreted through the same framework of metabolic suppression developed in RWC discussion. *K. sacchari*'s contribution to shoot elongation would be expected to operate through IAA mediated stimulation of cell elongation and root development and through the supply of fixed nitrogen to support growth processes (Glick, 2012; Romano et al., 2020). Both mechanisms depend on active bacterial metabolism in the root endosphere. As argued in section 5.2, drought-induced osmotic stress and ROS accumulation in the root apoplast are likely to have suppressed nitrogenase activity, and redirected bacterial energy towards osmoprotectants synthesis, effectively rendering *K. sacchari* growth-promoting inactive during the stress period. This is consistent with the evidence that osmotic stress impairs nitrogenase activity and triggers preferential osmoprotectants accumulation, including glutamate and trehalose in diazotrophic bacteria as a survival response. Madkour et al. (1990) and with finding that water deprivation causes a significant decrease in nitrogenase activity in diazotrophic bacteria associated with the host plants (Defez et al., 2017b).

The negative shoot length response observed in inoculated Vialone Nano plants is consistent with the existing literature. Andreozzi et al (2019) worked with the same *K. sacchari* RCA25 reported that Vialone Nano showed no significant improvement in shoot growth and in some parameters even a negative response-following inoculation, while the cultivar Baldo showed strong shoot dry weight and nitrogen content. An explanation for the absence of inoculation-mediated shoot growth promotion across all the genotypes lies in the disruption of the carbon supply underlying the plant-endophyte interactions. Endophytic diazotrophs such as *K.sacchari* depend on photosynthetically derived carbon compounds such as sugars and amino acids in exchange for fixed nitrogen (Puri et al., 2018).

Under drought stress, photosynthetic rate declines and stomata close (Chaves et al., 2002; Flexas and Medrano, 2002), and the carbon available for export to the root endosphere is substantially reduced (Kuzyakov and Gavrichkova, 2010). This has been demonstrated in legumes-rhizobia systems, where drought inhibits sucrose synthase activity in nodules and decreased malate concentrations, thereby starving bacteroids of the carbon substrate required for nitrogenase function (Ladrera et al., 2007). Although *K.sacchari* is a free-living endophyte rather than a nodule-forming symbiont, the same carbon-limitation principle applies, nitrogen fixation require

a minimum of 16 ATP per N<sub>2</sub> molecules fixed under ideal conditions (Burgess and Lowe, 1996), and this energetic demand cannot be sustained when host plant's carbon budget is already diverted towards osmotic adjustment and ROS detoxification (Miller et al., 2010).

This carbon reallocation may be particularly pronounced in young seedlings, whose limited photosynthetic area provides a smaller carbon reserve than mature plants. Uninoculated control plants face no such demand on their carbon output, all photosynthate is channelled directly into growth which may explain why controls consistently achieved greater shoot elongation across all genotypes, even under well-watered conditions. Rather than reflecting a failure of *K.sacchari* RCA25 to colonise or function, these results may therefore reflect a developmental and stress-content specific suppression of the plant endophyte mutualism, a phenomenon increasing recognised in the PGPB literature.

Future studies should test inoculation responses across a wider range of stress intensities and durations. It would also be valuable to explore whether using bacteria in combination produces stronger and more consistent results. Rice inoculated with a consortium of *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* has already been shown to produce better responses to salinity stress compared to single strain inoculation, suggesting that complementary bacterial partnerships may be a more effective strategy than relying on a single endophytic strain (Jha and Subramanian, 2011).

#### **5.4 RNA Integrity and Quality Control**

Gel electrophoresis of Batch 1 samples revealed clear differences in RNA quality across genotypes (Figure 14). The introgression lines IL189 and IL273 produced the most consistent results, showing two well-defined ribosomal RNA bands, corresponding to 28S and 18S subunits in a ratio of 2:1, with a low molecular weight RNA visible at the bottom of the gel (Figure 18). Even the trial samples, which contained a smaller amount of starting material, showed visible bands with slightly reduced intensity compared to the main IL samples (Figure 19), confirming the effectiveness of the extraction method used.

Trizol based extraction was selected as the primary extraction method because it is a well-established, single reagent protocol capable of disrupting cells, denaturing proteins, and maintaining RNA stability during phase separation (Chomczynski and Sacchi, 1987). TRIzol

contains guanidinium thiocyanate and phenol which inactivate RNases enzymes at the point of cell lysis providing protection of RNA integrity (Chomczynski and Sacchi, 1987). DNase treatment was applied to all samples to eliminate residual genomic DNA contamination, which can generate false signals during PCR based quality control checks or introduce artefacts in RNA-seq libraries (Williams et al., 2014). The combination of TRIzol extraction with DNase digestion provided a robust two-step approach to obtaining RNA that is both intact and free from DNA contamination as confirmed by the absence of higher molecular weight DNA bands above the 28S rRNA on the agarose gel (Figure 18). The clear 28S and 18S bands observed in the ratio 2:1 are the primary visual indicators of intact total RNA, as intact ribosomal RNA produce these two dominant peaks (Bio-Rad Laboratories, 2008)

In contrast to the IL samples, the parental lines *O. rufipogon* and Vialone Nano show variables and in several cases severely compromised results in Batch 1. Initial gels displayed heavy smearing across the lanes (Figure 20), a pattern indicative of RNA degradation where intact ribosomal bands are replaced by a continuous smear of fragments molecules migrating at lower molecular weights (Schroeder et al., 2006). The Bioanalyzer analysis confirmed these observations, assigning RIN values  $\leq 7$  to most of Batch 1 parental line samples- below the widely accepted minimum threshold for RNA-seq (Schroeder et al., 2006).

Low RIN has downstream consequences on RNA sequencing. RNA degradation introduces systemic bias into RNA-seq libraries prepared by poly-A selection, as transcript-level degradation rates are significantly correlated with CDS length, 3' UTR length, and GC content (Romero et al., 2014). This means that longer transcripts and those with complex isoforms are disproportionately affected, leading to uneven gene body coverage and underestimation of expression levels for affected genes (Romero et al., 2014). Low and variable RIN across samples introduces systemic noise into gene expression quantification. Schroeder et al. (2006) established that degradation reduces library complexity, inflating apparent differences between samples. Comparing samples with different RIN values is problematic because degraded RNA over-represents a small number of highly expressed genes, distorting relative transcript abundances (Romero et al., 2014). Including RIN as a covariate in downstream differential expression models ( e.g. DESeq2 or edgeR linear models) can recover biologically meaningful signal even when some degradation has occurred (Romero et al., 2014)

The degradation observed in Batch 1 parental line samples was most likely caused by partial thawing of root tissue during grinding step, which would permit reactivation of endogenous RNases prior to complete cell lysis- rather than any intrinsic property of the tissue itself This interpretation is supported by the fact that both Vialone Nano and *O.rufipogon* were affected, and that Batch 2 improvements specifically faster tissue lysis, greater liquid nitrogen volume and tighter temperature control throughout the extraction directly address the kinetics of RNase activity, which is highly time and temperature-dependent (Chomczynski and Sacchi, 1987).

Batch 2 results confirmed that these protocol modifications were effective. *O.rufipogon* samples produced clear 28S and 18S bands with no DNA contamination and no visible smearing, representing the best gel results obtained for this genotype throughout the study (Figure 25). Vialone Nano control samples were also showed clear bands although Vialone Nano treated samples exhibited some partial smearing despite high RNA concentrations, suggesting residual handling variability in this genotype. All Batch 2 samples achieved concentrations above 20 ng/ $\mu$ L by Qubit quantification and Bioanalyzer analysis confirmed that the majority of *O.rufipogon* and Vialone Nano samples achieved RIN values  $\geq 7$ , representing a clear improvement over Batch 1. All samples meeting the quality threshold have been submitted to IGA Technology Services (Udine, Italy) for RNA-seq library preparation; sequencing is currently under way and differential gene expression analyses will be conducted upon receipt of the data.

## CONCLUSION AND FUTURE PESPSECTIVES

### 6.1 Conclusion

This thesis investigated the association between rice introgression lines (ILs), derived from *Oryza sativa* cv Vialone nano × *O.rufipogon* 602-131-2 (BC<sub>3</sub>F<sub>5</sub>), and the endophytic bacterium *Kosakonia sacchari* RCA25, examining root colonisation, drought stress responses, and the transcriptomic readiness of selected genotypes.

Screening of 41 ILs and their parental lines revealed significant genetic variation in the capacity to support RCA25 root colonisation, with mean log<sub>10</sub> CFU/g values ranging from 6.07 to 8.17 across the panel. IL36 and IL28 recorded the highest colonisation levels, while IL8 and IL6 recorded the lowest. The significantly higher colonisation observed in *O.rufipogon* (8.23 log<sub>10</sub> CFU/g) relative to Vialone Nano (7.29 log<sub>10</sub> CFU/g) confirms that host genotype is a primary determinant of colonisation efficiency. These results support H1 that introgression lines exhibit differential colonisation relative to the recipient parent and H2 that the wild parent *O.rufipogon* sustains higher colonisation than the elite cultivar, consistent with a domestication-associated erosion of endophyte recruitment capacity. The dispersion of colonisation phenotypes across the IL panel is consistent with endophyte affinity being a quantitative trait controlled by multiple *O.rufipogon*-derived loci.

Physiological assessment of four selected genotypes under drought stress, with and without RCA25 inoculation, revealed genotype-dependent responses in both shoot length and relative water content (RWC). No statistically significant inoculation effect on RWC was detected (GLM: treatment,  $p = 0.241$ ). For shoot length, a three-way GLM identifies Genotype ( $p < 0.001$ ), Treatment ( $p < 0.007$ ) as source of variation, confirming that inoculation effects were genotype-dependent. Across all genotypes, uninoculated controls consistently outperformed inoculated plants, with Vialone Nano showing the most pronounced suppression of shoot elongation under inoculation. These results do not support H3; the absence of a stress-protective effect is attributed to drought-induced suppression of bacterial nitrogenase activity, reduced host carbon availability to the endophyte, and the absence of ACC deaminase activity in *K.sacchari*. Regarding H4, the genotype-dependent transcriptional responses to bacterial colonisation under drought stress could not be assessed within the scope of this thesis, as RNA sequencing data are currently awaited; this remains a primary objective of the broader researcher programme

RNA extraction from 48 samples root tissue samples demonstrated that TRIzol-based extraction with DNase treatment reliably produced high-quality RNA from IL samples. Batch 2 samples yielded a marked improvement of the RNA quality, with the majority of *O.rufipogon* and Vialone Nano samples achieving RIN values  $\geq 7$  with peak values reaching 9.5-confirming their suitability for RNA sequencing. Together, these results establish a high-quality RNA sample collection ready for downstream transcriptomic analysis to elucidate the molecular mechanisms underpinning genotype-dependent plant-endophyte interactions under drought stress. Sequencing is currently underway at IGA Technology Services (Udine, Italy).

## 6.2 Future Perspectives

The colonisation screen should be expanded to the full IL population to capture the complete spectrum of genetic variation influencing RCA25 recruitment. Future studies should also assess whether varying bacterial inoculum concentration-defined by optical density (OD) thresholds at the time of inoculation-affects colonisation efficiency in a dose-dependent manner. Establishing whether a minimum or optimal inoculum concentration exists for consistent endophytic establishment would substantially improve experimental reproducibility and allow more meaningful comparisons across genotypes and experiments. Standardised inoculation protocol incorporating defined OD thresholds are therefore a prerequisite for scaling these experiments to larger IL populations.

Comparative screening using multiple endophytic species, and exploration of Synthetic Microbial Community (SynCom) approaches, would clarify whether multi-strain inoculant outperform single-strain inoculation for colonisation and plant growth promotion. The drought stress component would benefit from direct quantification of biological nitrogen fixation through leaf nitrogen content analysis and  $^{15}\text{N}$  isotope dilution assays, which would provide more direct evidence of whether *K. sacchari* nitrogen-fixing activity remains functional under osmotic stress. Expanding the number of genotypes tested and transitioning to hydroponic systems would provide more controlled conditions and eliminate confounding variability inherent to soil-based experimental designs.

For RNA quality control, future protocols should incorporate NanoDrop spectrophotometry alongside gel electrophoresis and Bioanalyzer analysis as standard practise, providing complementary assessment of proteins and solvent contamination that can inhibit library

preparation even when gel results appear acceptable. Once RNA sequencing data are returned, integration with the genomic map of introgressed *O.rufipogon* segment in IL273 and IL189 will enable identification of candidate genomic regions underlying variation in endophyte recruitment and stress tolerance. Furthermore, the phenotypic colonisation data generated across the 41 ILs in this study and the DNA already extracted from these lines will provide a dataset for future quantitative trait locus (QTL) mapping across the broader population of introgression panel. Linking colonisation phenotypes and stress responses parameters to specific *O.rufipogon* derived genomic will facilitate the identification of genes and regulatory pathways governing endophyte recruitment and drought resilience, ultimately contributing to more targeted and sustainable rice breeding strategies that leverage beneficial plant-microbe interaction for improved performance under abiotic stress conditions increasingly associated with climate change.

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