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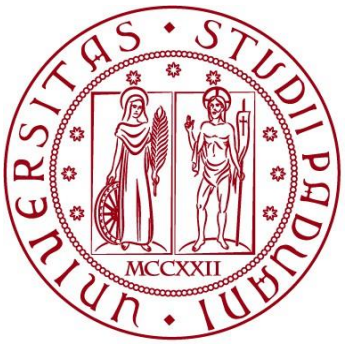

TESI DI LAUREA

Investigating the Role of Compost Microbiota for Improving Plant Health and Resistance to Stress

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1. Introduction

1.1 Soil Biodiversity

A great diversity of life inhabiting soil in the form of living organism of different taxa and genes, as far as their mutual contribution at the level of ecological systems and biotic landscapes, can be found in every microscopic soil particle and covers almost all the landscapes on the earth. This plethora of life, almost unseen in comparison with above-ground organisms and often considered of less significance, is actually underlying a lot of the processes that enable food production as well as soil and water purification.

Every fourth species contributes to the planetary soil biodiversity. This group includes the entire range of living beings, including those typical of upper horizon biomes, such as bacteria, archaea, fungi, algae, and protozoa; invertebrates such as nematodes; insect larvae; earthworms; arthropods and their larval stages; and mammals, reptiles, and amphibians that are primarily burrowing animals (GSBI, 2020).

1.1.1 The importance of soil biodiversity to ecosystem services

Ecosystem nutrients and their cycling, soil formation and food web stabilization are profoundly provided through the biodiversity in the soil. These services help conserve and harness the biodiversity in the ecosystem. Among the key functions of soil biodiversity are:

- Food, fiber, and fuel production.
- Water filtration.
- Source of molecules with industrial and/or pharmaceutical interest.
- Carbon and nutrient cycles.
- Soil formation.
- Mitigation of greenhouse gases.
- Pest and disease control.
- Decontamination and remediation.

Soil biodiversity is the main responsible of the conversion of organic and inorganic molecules, in the form which can be accessed by plants among other organisms. These processes are also referred to as nutrient cycling and organic matter decay (FAO, 2022; GSBI, 2020; Philippot, 2023).

1.1.2 Soil biodiversity loss

The diversity of soil communities is threatened by a suite of human-made and natural processes, many of which are exacerbated by the changing climate caused by humans. One of the principal global challenges is facing the consequences of climate change and addressing the solutions necessary to reduce the impact of the so called 'Anthropogenic changes'. The main anthropogenic threats to soil biodiversity can be resumed in few points:

- Deforestation: leads to loss of soil organic matter and nutrients with consequent changes in soil physical characteristics.
- Soil compaction: caused by heavy machinery used in agriculture or overgrazing, reduces soil porosity, restricting the movement of air, water and nutrients, and adversely affecting microbial activity.
- Erosion and landslides: the loss of topsoil due to erosion reduces the amount of habitat available for soil organisms and can lead to the dispersal of harmful organisms.
- Fires: fires damage soil biodiversity, especially in the surface layers, and can alter the composition of the microbial community.

(FAO, 2022; GSBI, 2020; Hartmann and Six, 2023)

1.1.3 The Importance of Sustainable Soil Management

Taking care of the soil in a sustainable way is significant for securing its biodiversity and the environmental services it provides. Different strategies can be adopted to reduce the loss of biodiversity:

- Reducing soil disruption: techniques like direct seeding or minimal tillage help maintain soil structure, increase organic matter, and promote microbial diversity.
- Diversifying crops: crop rotation, intercropping (developing diverse plants together), and agroforestry are effective ways to keep the soil healthy, sustaining microbial diversifications, and minimizing pathogen problems.
- Using natural fertilizers: natural fertilizers, like composted fertilizer, can be considered not only to help soil structure and as a source of organic matter but also to supply fundamental elements for soil life forms and new living microorganisms.

- Managing crop residues: the preservation of crop residues on the soil surface can protect the soil from erosion, improve water infiltration and promote the decomposition of organic matter.
- Integrated pest and disease control: Integrated Pest Management (IPM) uses a combination of methods to minimise pesticide use, preserving natural enemies of pests and promoting soil biodiversity.

(FAO, 2022; GSBI, 2020; Hartmann and Six, 2023)

1.1.4 Challenges and opportunities for research and policy

Despite the developing awareness of the significance of soil biodiversity, numerous challenges remain to be faced including:

- Increase information on soil biodiversity: the differences in the soil microbiome are still generally unexplored. Advance considerations are required to characterize the composition, dissemination, and capacities of soil biota in diverse ecosystems.
- Establish standardized indicators and methods: the lack of standardized indicators and methods for assessing soil biodiversity makes it difficult to compare data across studies and regions. Efforts are needed to harmonize sampling and analysis techniques to gain a more complete view of soil biodiversity worldwide.
- Considering soil biodiversity during decision processes: soil biodiversity is only occasionally considered conservation and restoration programs. It is vital to promote the integration of soil biodiversity into national and universal approaches to guarantee its security and feasible use.
- Raising public awareness: most individuals are not aware of the significance of soil biodiversity and its environmental services. Awareness-raising campaigns are required to teach the open almost the significance of securing soil life.

Soil biodiversity represents an opportunity to create nature-based solutions to numerous worldwide challenges, such as food security, climate change and human well-being. (FAO et al.2022; GSBI, 2020).

1.2. Soil Microbiome

The soil microbiome, comprising the totality of microorganisms dwelling in the soil, speaks to a biological system of exceptional complexity and significance.

Although imperceptible, this minuscule world plays a principal part in keeping up soil wellbeing, impacting plant development, nutrient cycling, climate control and human wellbeing. The soil microbiome is not inactive, but changes with natural conditions, agricultural practises and other variables. Its understanding is vital for the advancement of sustainable procedures for soil management and food production (FAO, 2022).

1.2.1 Composition of the Soil Microbiome

The soil microbiome is a mosaic of distinctive life shapes, each with its own particular part in the soil's biological system. The primary components include:

- Microorganisms: bacteria and fungi represent the most varied and differing parts of the soil microbiome. They perform a wide range of functions, from breaking down organic matter to fixing atmospheric nitrogen. Their abundance is influenced by factors such as soil pH, nutrient availability, and moisture. Among the most important groups of soil microorganisms are bacteria, which are the most abundant in the soil and play a crucial role in processes like organic matter decomposition, nutrient cycling, and nitrogen fixation. Notable examples of bacteria include denitrifying bacteria, nitrogen-fixing bacteria, and ammonia-oxidizing bacteria. Another important group is archaea, unicellular microorganisms that often thrive in extreme environments. In the soil, they contribute to nutrient cycling, especially in the nitrogen cycle, with ammonia-oxidizing archaea being a key example. Fungi are also essential, as they help decompose organic matter, form soil structure, and cycle nutrients. They can be classified according to their ecological function, such as symbionts, saprotrophs, and pathogens. Algae, photosynthetic microorganisms found primarily in the surface layers of the soil, contribute to primary production and the carbon cycle. Lastly, protozoa are unicellular eukaryotes that feed on bacteria, fungi, and other protozoa. They regulate microbial populations and release nutrients that are important for plant growth. (FAO, 2022; Jansson, 2023). Bacteria and fungi are fundamental decomposers as they can degrade natural polymers such as lignin and cellulose. A few parasites set up advantageous connections with plant roots, which help nutrient and water uptake (GSBI, 2020; Kumar, 2020; Hartmann and Six, 2023).

- Archaea: although less examined than prokaryotes and fungi, archaea play a critical part in forms such as the nitrogen cycle and methane generation. They are frequently found in extreme situations, such as saline or acidic soils (FAO, 2022).
- Protists: they are unicellular eukaryotic organisms that feed on bacteria, fungi and other microorganisms, regulating the soil microbial population (FAO, 2022; Jansson, 2023).
- Viruses: they infect bacteria, archaea, fungi and protists, influencing microbial population dynamics and gene flow in the soil.

The composition of the soil microbiome is influenced by numerous factors, including:

- Land use: the conversion of forests to agricultural land, for example, can significantly alter the composition of the soil microbiome.
- Agricultural practices: soil tillage, fertilisation and pesticide use can influence the diversity and abundance of soil microorganisms (FAO, 2022).
- Soil properties: soil pH, texture, structure and organic matter content are key factors influencing the composition of the soil microbiome (Hartmann and Six, 2023).
- Climate: temperature, precipitation and other climatic factors influence the growth and activity of soil microorganisms (FAO, 2022).

1.2.2 Functions of the Soil Microbiome

The soil microbiome is involved in many fundamental processes, and it participates in the basic cycles of nutrients and molecules useful for plant nutrition. Among many, the main soil microbiome capacities include:

- Nutrients cycles: soil microorganisms are at the base of the biogeochemical cycles of fundamental components such as carbon, nitrogen, phosphorus, and sulphur. Microorganisms break down organic matter, transforming mineral nutrients in chemical types accessible to plants. A few bacterial species capture atmospheric nitrogen, transforming it into nitrogen salts, a shape of nitrogen usable by plants (FAO, 2022; GSBI, 2020; Hartmann and Six, 2023; Jansson, 2023).
- Decay of organic matter: soil microorganisms are the primary decomposers of organic matter, transforming material from plants and organisms into less complex compounds, releasing nutrients and

contributing to the development of humus. The rate of deterioration is impacted by the composition of the soil microbiome, environmental conditions, and the quality of the natural matter (Kumar and Meena, 2019; FAO, 2022; Hartmann and Six, 2023).

- Soil structure: soil microorganisms deliver substances that tie soil particles, contributing to the arrangement of steady totals, progressing soil structure and its capacity to hold water and nutrients (Philippot, 2023; Hartmann and Six, 2023).
- Suppression of pathogens: a few soil microorganisms create anti-microbials or compete with plant pathogens for nutrients and space, contributing to infection control. The differences of the soil microbiome are critical for illness resistance, as a more diversity of microorganisms gives a more effective defense line (Arora & Bouizgarne, 2022; FAO, 2022; Hartmann and Six, 2023).
- Climate direction: soil microorganisms impact the carbon cycle by discharging carbon dioxide (CO_2) into the environment through emission of greenhouse gases, and sequestering carbon in the soil in as organic matter. Soil management can influence CO_2 fluxes from the soil. (FAO, 2022; Hartmann and Six, 2023).

The soil microbiome also impacts human wellbeing in different ways. Soil microorganisms are a source of anti-microbials and other bioactive compounds. Soil wellbeing is connected to food security, as soil microorganisms influence and improve plant development, nutrients quality, and spreading of contaminants (Arora & Bouizgarne, 2022; FAO, 2022).

The soil microbiome is a complex and energetic biological system that plays a principal part in keeping up soil wellbeing and supporting life on Soil. Its composition and capacities are impacted by many components, counting soil administration hones. Understanding the soil microbiome is basic for creating maintainable methodologies for nourishment generation, moderating climate alteration, and defending human wellbeing.

1.3. The Rhizosphere and its microbiome

The rhizosphere is the zone of soil surrounding plant roots, where many interactions among soil bacteria and roots take place. It is a dynamic and complex environment, the interface between plant roots, microorganisms, and the soil network. Inside the rhizosphere, the rhizosphere microbiome incorporates bacterial communities and compete in this interesting living space. Rhizosphere microorganisms play a pivotal role in plant health and

efficiency, impacting mineral nutrition, pathogen responses, and plant development (Philippot, 2013).

1.3.1 Composition of the Rhizosphere Microbiome

The rhizosphere offers a nutrient-rich environment for microorganisms, thanks to the presence of root exudates, cell debris, and mucilage. This richness of molecules allows a wide variety of bacterial taxa, driving to higher bacterial colonization and metabolisms in the rhizosphere compared to the bulk soil (Philippot, 2013). The composition of the bacterial community in the rhizosphere is not randomly defined, but is shaped and influenced by different factors, including:

- Soil type: the physico-chemical composition of the soil, pH, structure, and nutrient substances, essentially impacts the composition of the soil microbiome and, subsequently, the rhizosphere fungal and bacterial communities.
- Plant species and genotype: different plant species produce different root exudates that establish a particular subset of rhizosphere microscopic organisms. Indeed, inside the same plant species, diverse varieties or genotypes can have diverse rhizosphere bacterial communities.
- Plant formatives organize: the composition of rhizosphere community changes according to the plant development stage, reflecting changes in root exudates and the specialty situations given by diverse root zones.
- Environmental conditions: variables such as temperature, level of precipitations, and the nearness of environmental contaminants moreover impact the composition of rhizosphere communities.

Although distinctive bacterial species can be found in the rhizosphere, a few bacterial taxa reliably rule this environment. Proteobacteria, especially those having a place to the *Pseudomonadaceae* and *Burkholderiaceae* families, are regularly the most copious individuals of the rhizosphere microbiome. These microorganisms are by and larger strategists, characterized by quick development and the capacity to utilize a wide run of carbon substrates exudate from roots. Other bacterial taxa as often as possible experienced in the rhizosphere incorporate *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Philippot, 2013; Arora & Bouizgarne, 2022).

The bacterial biodiversity in the rhizosphere essentially contributes to the multifunctionality of this biological system, and it has been demonstrated that rich bacterial communities perform an assortment of metabolisms and activities advantageous to plants (Philippot, 2013).

1.3.2 Signalling Between Plants and Bacteria

The rhizosphere is a location of strong chemical communication between plants and microorganisms. Plants release a wide extend of compounds through their roots, collectively known as root exudates, which are recognized as signals by rhizosphere microorganisms. These signals can give advantages to some bacterial or fungal taxa, contrast pathogens, or regulate bacterial physiology. Rhizosphere microorganisms, in turn, can produce and release signalling molecules that can impact plant development and growth, as well as defense reactions. This complicated network of chemical communication is fundamental for building up and keeping up advantageous plant-microbe connections (Mohamed, 2021; Philippot, 2013).

1.3.3 Rhizosphere Selection Process

The composition of the rhizosphere bacterial community is influenced by many factors. The process of community assembly is guided by a complex interaction of components, counting plant root exudates, plant resistance reactions, and competitive metabolisms among microorganisms (Hakeem & Akthar, 2016; Mohamed, 2021; Arora & Bouizgarne, 2022).

1.3.4 Recruitment of Beneficial Bacteria

Plants can effectively recruit advantageous microorganisms in their rhizosphere by releasing signals through their root exudates. These signals can attract bacterial species that give advantages to the host plants: helping mineral nutrition, competing with pathogens, and, in general, enhancing plant growth. For example, plants may selectively attract phosphate-solubilizing microorganisms, making this basic nutrient more accessible to the plant and, plants can recruit species that are able to produce anti-microbials or siderophores, which help controlling pathogens and improve iron uptake (Hakeem & Akthar, 2016; Mohamed, 2021; Arora & Bouizgarne, 2022; Kumar, 2020; Sayyed, 2019).

1.3.5 Plant Immune Responses

Plants have a complex array of receptors and molecular pathways that permits them to recognize and react to organisms, counting rhizosphere microorganisms. Plant resistant reactions can shape the composition of the rhizosphere bacterial community by selecting against possibly harmful microorganisms, and helping the establishment of useful ones. Plants can identify organisms by recognizing microbe-associated atomic designs (MAMPs), which are conserved domains exposed on the surface of organisms. The recognition of MAMPs triggers the activation of defense reactions in plants, counting the generation of responsive oxygen species (ROS), reinforcing cell walls, and producing antimicrobial secondary metabolites. These defense reactions can hinder the development of certain rhizosphere microscopic organisms, whereas other advantageous microscopic organisms have created instruments to sidestep or smother plant resistance reactions (Hakeem & Akthar, 2016; Mohamed, 2021; Philippot, 2013).

1.3.6 Competitive Interactions Among Bacteria

Competition among rhizosphere microscopic organisms moreover plays a noteworthy part in shaping community composition. Bacteria compete for limited resources, such as nutrients and space, and may synthetize antimicrobial compounds to repress the development of competitors. Useful microorganisms can outcompete pathogens for resources or deliver antimicrobial compounds that inhibit pathogens. Plants can modulate this competition by modulating root exudates composition to favour the development of beneficial microorganisms (Hakeem & Akthar, 2016; Mohamed, 2021; Kumar, 2020).

1.4. Plant Growth-Promoting Rhizobacteria

Plant Growth-Promoting Rhizobacteria (PGPR) are a group of beneficial bacteria that inhabit the rhizosphere, the region of soil surrounding plant roots. These bacteria have a profound influence on plant growth and health through various direct and indirect mechanisms. PGPR enhance plant growth by improving nutrient availability, modulating plant growth processes, and providing protection against diseases (Kumar and Meena, 2019).

1.4.1 Nutrient Availability Improvement

PGPR play an essential part in making essential nutrients accessible for plant uptake. They can do this through different mechanisms:

- Nitrogen Fixation: nitrogen is a basic nutrient for plant development, but plants cannot specifically utilize air nitrogen. A few PGPR, known as nitrogen fixers, have the protein nitrogenase, which permits them to transform gaseous nitrogen into nitrates, a type of nitrogen usable by plants. Illustrations of nitrogen fixers incorporate *Azotobacter*, *Azospirillum, Rhizobium, and Bacillus* (Kumar, 2020; Kumar and Meena, 2019; Sayyed, 2019).
- Phosphate Solubilization: phosphate is another fundamental nutrient for plant development, but it is frequently present in the soil as insoluble salts that plants cannot absorb. PGPR can solubilize insoluble phosphate by producing natural acids, chelators, and phosphatases. These molecules change over phosphate into soluble shapes that plants can effectively assimilate. Bacterial genera such as *Pseudomonas, Bacillus and Rhizobium* are known for their phosphate solubilization capabilities (Kumar, 2020; Kumar and Meena, 2019; Sayyed, 2019; Hakeem & Akthar, 2016).
- Siderophore Generation: iron is a basic micronutrient for plant development, but its accessibility in the soil is frequently constrained. PGPR can produce siderophores, which are high-affinity molecules for iron. Siderophores chelate ferric iron, making it accessible for assimilation by plants whereas moreover restricting the accessibility of press for pathogens. Illustrations of siderophore-producing PGPR incorporate *Pseudomonas and Bacillus* (Arora & Bouizgarne, 2022; Kumar, 2020; Kumar and Meena, 2019).

In conclusion, PGPR plays a vital role in enhancing nutrient absorption in plants by employing multiple mechanisms that contribute to overall plant health and resilience. By expanding the root surface area and stimulating robust root growth, PGPR increases the plant's ability to access and absorb essential nutrients. Additionally, these beneficial bacteria improve the activity and efficiency of nutrient transport proteins, ensuring that nutrients are effectively moved within the plant. Furthermore, PGPR influences root architecture, allowing plants to explore soil more thoroughly and make the most of available resources. Together, these effects lead to stronger, healthier plants with improved nutrient uptake and growth potential (Kumar and Meena, 2019; Hakeem & Akthar, 2016).

1.4.2 Phytostimulation

PGPR can specifically improve plant development by producing phytohormones, which are chemical controllers of plant growth. Bacteria are known to have metabolic pathways for:

- Auxin: a phytohormone that plays a role in cell division, cell prolongation, and root development. Numerous PGPR can produce auxins, especially indole-3-acetic acid (IAA). The generation of IAA by PGPR can promote root development, increase nutrient take-up, and generally promote plant growth. *Bacillus, Pseudomonas and Rhizobium* are known IAA producers (Kumar and Meena, 2019; Hakeem & Akthar, 2016; Sayyed, 2019).
- Cytokinins: a group of phytohormones that promote cell division, bud separation (this refers to the formation and separation of new growth buds, which can develop into branches, leaves, or flowers; cytokinins promote this process by stimulating the production of new growth points), and delay senescence. A few PGPR can deliver cytokinins, which can contribute to regulate and promote plant development, bud improvement, and expanded stretch resistance (this refers to the plant's enhanced ability to resist physical stress, such as stretching or pulling from wind, rain, or manipulation, and maintain its structure under such conditions) (Sayyed, 2019).
- Gibberellin: phytohormones that control stem elongation, seed germination, and flowering. Some PGPR can deliver gibberellins, which can stimulate plant elongation, biomass production, and modulate flowering (Sayyed, 2019).
- Modulation of Ethylene: ethylene is a gas used by plants as phytohormone, and involved in, senescence, and stress reactions. High levels of ethylene can have inconvenient impacts on plant development. A few PGPR can produce the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which degrades ACC, the precursor of ethylene. By lowering ethylene levels in the roots, these PGPR can moderate the negative impacts of environmental stresses, promoting plant growth. The ACC deaminase production has been recorded in numerous plant growth-promoting species under different conditions (Kumar and Meena, 2019; Arora & Bouizgarne, 2022).

1.4.3 Pathogen Control

PGPR can reduce plant infections through different mechanisms protecting plants and sustaining plant growth. The main mechanisms of plant biocontrol by PGPR are:

- Production of antibiotics: PGPR produce anti-microbials, which are compounds that repress or inhibit other microorganisms, including plant pathogens. Cases of anti-microbials delivered by PGPR include 2,4-diacetylfluoroglucinol, phenazine-1-carboxylic acid, pyrrolnitrin, and pyoluteorin. *Pseudomonas* and *Bacillus* are known for their anti-microbial molecules (Kumar and Meena, 2019; Arora & Bouizgarne, 2022; Sayyed, 2019).
- Competition: PGPR compete with pathogens for nutrients and space in the rhizosphere, restraining the development and expansion of these pathogens (Kumar and Meena, 2019; Sayyed, 2019).
- Lytic Enzymes: a few PGPR create lytic proteins, such as chitinases, glucanases, and proteases, which can damage the cell of pathogens (Kumar and Meena, 2019; Arora & Bouizgarne, 2022; Sayyed, 2019).
- Induced Systemic Resistance (ISR): PGPR can activate plant defenses, a process known as ISR. ISR is a state of increased protective preparation that primes plants for pathogen assaults. PGPR that initiate ISR trigger signalling pathways in plants that lead to the accumulation of defense-related proteins, fortifying of cell walls, and production of antimicrobial secondary metabolites (Kumar and Meena, 2019; Arora & Bouizgarne, 2022; Sayyed, 2019).

PGPR are essential protagonists of the rhizosphere microbiome and play a principal part in promoting plant development and health. Their different instruments of activity, including improving nutrient accessibility, phytohormone generation, and pathogen control, make them promising candidates for creating sustainable agricultural approaches. By understanding the complex interaction between PGPR and plants, we can tackle their potential to improve plant efficiency, decrease dependence on chemical fertilizers and pesticides, and advance sustainable agricultural practices.

1.5. Threats to Microbiome Biodiversity

The plant microbiome, which includes an assortment of microorganisms living inside and around plants, plays a vital part in plant wellbeing and efficiency (Arora & Bouizgarne, 2022; Compant, 2024). These microorganisms contribute

to a range of essential processes, such as nutrient acquisition, tolerance to both biotic and abiotic stresses, and overall plant growth and development.

However, the biodiversity of the microbiome is progressively threatened by a extend of components, including climate change and intensive agricultural practices (Bakhshandeh, 2019; Compant, 2024; FAO, 2022). These threats can have significant consequences for agricultural ecosystems, leading to reduced crop productivity, increased susceptibility of plants to diseases, and a decline in soil quality.

Understanding the threats to microbiome biodiversity and creating procedures to relieve them is essential for guaranteeing the long-term sustainability of agricultural systems (FAO, 2022; Arora & Bouizgarne, 2022).

1.5.1 Drought and its stress in plants

Among all the adverse environmental conditions that plants may face, drought is one of the primary causes of abiotic stress in plants. health (Arora & Bouizgarne, 2022).

Lack of available water in the soil leads to a cascade of physiological reactions in plants aimed at reducing the effects of water loss. Some of the direct effects on plants are:

- Reduced water absorption.
- Stomatal closure.
- Decreased photosynthesis.

Low availability of water and plant physiological changes influence the plant microbiome in different ways:

- Modifications in Root Exudates: plants under drought stress change the composition and amount of root exudates, which are the primary source of nutrients for rhizosphere microorganisms. This can lead to changes in the composition and diversity of the microbial community (Arora & Bouizgarne, 2022; FAO, 2022).
- Reduction in Soil Moisture: drought decreases soil moisture, which influences the survival and activity of soil microorganisms (Arora & Bouizgarne, 2022; FAO, 2022).
- Increased Oxidative Stress: drought increases the production of reactive oxygen species (ROS) in plants, which can harm both plant cells and soil microorganisms (Arora & Bouizgarne, 2022).

The combined effects direct to the plant and to the plant microbiota have negative results for plant health and productivity (Arora & Bouizgarne, 2022), these effects lead to reduced nutrient uptake and increased susceptibility to diseases, ultimately resulting in decreased growth and yield.

1.5.2 Environmental changes

In addition to drought, other natural changes, such as rising temperatures, pollution, and intensive agricultural practices, pose a critical threat to microbiome biodiversity (Arora & Bouizgarne, 2022; Bakhshandeh, 2019; FAO, 2022).

1.5.2.1 Impact of Environmental Changes on the Plant Microbiome

Various natural changes can impact the plant microbiome, including:

- Rising temperatures: increased temperatures can change the composition and function of soil microbial communities, favouring thermophilic microorganisms and compromising those sensitive to heat (Arora & Bouizgarne, 2022).
- Pollution: contamination from heavy metals, pesticides, and other contaminants can have toxic effects on soil microorganisms, reducing their diversity and abundance (FAO, 2022).
- Intensive agricultural practices: heavy agricultural practices, such as tillage, excessive use of fertilizers and pesticides, and monocultures, can negatively affect microbiome biodiversity (FAO, 2022).

1.5.2.2 Consequences for Agricultural Ecosystems

The alteration of the plant microbiome due to natural and anthropogenic changes can have far-reaching consequences for agrarian environments (FAO, 2022):

- Decreased Soil Fertility: the loss of beneficial soil microorganisms can reduce soil fertility and its capacity to support plant growth.
- Increased Greenhouse Gas Emissions: changes in the soil microbial community can influence biogeochemical cycles, driving increased emissions of greenhouse gasses such as carbon dioxide and nitrous oxide.

- Reduced Ecosystem Resilience: the loss of microbiome biodiversity decreases the resilience of ecosystems to natural changes and stresses, making them more vulnerable to degradation.

Microbiome biodiversity is fundamental for plant health and efficiency, as well as for the sustainability of agricultural environments. The dangers to microbiome biodiversity are a genuine concern and require urgent activity to mitigate them (Arora & Bouizgarne, 2022; FAO, 2022).

Future research should focus on (FAO, 2022):

- Gaining a better understanding of the effect of natural changes on the plant microbiome.
- Developing sustainable management strategies to protect and improve microbiome biodiversity.
- Promoting the development of microbiome-friendly agricultural practices, such as organic and conservation agriculture.

Protecting and upgrading microbiome biodiversity is critical for guaranteeing food security and long-term natural sustainability (FAO, 2022).

1.6. Methods used in the characterization of PGP bacteria

Plant Growth-Promoting Bacteria (PGPB) are a heterogeneous group of bacteria that can upgrade plant development through a variety of mechanisms. Characterizing PGP bacteria is fundamental for understanding their role in plant health and for developing techniques to improve their viability (Yi, 2018; Fan, 2011; Zhang, 2014).

1.6.1 Transformation with fluorescent proteins

Localization of beneficial bacteria on or within plant tissues is essential. For a complete understanding of the interaction between plants and microorganisms. Bacteria can be stained with specific stains or transformed genetically to obtain strains able to produce fluorescent proteins (FP). These proteins can fluoresce when exited by specific wavelengths and are crucial instruments for molecular biology and microbial ecology. They permit real-time tracking of bacteria in different environments, including plant tissues.

Advantages of Using FPs (Yi, 2018):

- FPs provide a non-invasive way to track bacteria.

- They can be utilized to study the colonization, development, and metabolic activity of living bacteria.
- Diverse FPs with varying emission spectra can be utilized to track numerous bacterial strains simultaneously.

To genetically transform environmental bacteria, genes encoding fluorescent proteins (FPs) can be introduced into bacteria through plasmids or chromosomal integration. While plasmids are easier to work with, they may be unstable, whereas chromosomal integration is more stable but harder to achieve. FPs can be expressed constitutively or under the control of inducible promoters, depending on the desired outcome (Magharbeh, 2021; Yi, 2018; Fan, 2011).

The performance of FPs can vary based on the bacterial host and the environment in which they are used, and it may be necessary to optimize FPs for enhanced brightness, stability, and compatibility with imaging methods. This optimization can be achieved through random mutagenesis and selection of variants with improved properties (Yi, 2018).

Examples of FPs commonly used in plant growth-promoting (PGP) bacteria include GFP (Green Fluorescent Protein), which is widely utilized with optimized variants available for various bacterial hosts; DsRed, a red FP that can be used alongside GFP for dual-colour labeling; mKate2, a bright and photostable red FP optimized for PGP bacteria; and sfGFP (Superfolder GFP), a GFP variant with improved folding and development, suitable for expression from weak promoters (Yi, 2018; Fan, 2011; Zhang, 2014).

1.6.2 Characterization of bacterial growth to understand kinetics

The characterization of the development of PGP bacteria is essential for understanding their physiology and behaviour under distinctive environmental conditions. The bacterial growth curve gives data about the different growth phases and kinetic parameters that can be used to compare strains and growth conditions (Fernandez-Martinez, 2024).

A typical growth curve of a bacterial strain has some distinctive phases (Kumakura, 2023; Fernandez-Martinez, 2024):

- Lag Phase: bacteria adjust to the new environment and get ready for growth.

- Exponential Phase: bacteria grow at a constant and fast rate. This stage is the most reproducible and permits direct comparisons between strains and conditions.
- Stationary Phase: growth stabilizes due to nutrient limitation.
- Death Phase: bacteria die due to extreme nutrient limitation.

Combining transformation techniques utilizing fluorescent proteins and growth characterization gives effective tools for studying PGP bacteria. In this thesis work we applied these methods to investigate plant-bacteria interactions, colonization, growth, and the factors influencing the effectiveness of PGP bacteria.

1.7 Aims of the Thesis

The primary objective of this thesis is to investigate and understand the interaction dynamics between plants and soil microorganisms, focusing on plant reactions to water stress conditions, and the impacts of different nutritional approaches on plant development and health. Through a series of experiments, this research points to enhancing the understanding of the mechanisms governing plant resilience in progressively challenging natural contexts, especially considering climate change and growing water scarcity. Besides the focus on drought stress, the research included a detailed characterization of bacterial strains isolated from compost. The differentiation in growth kinetics between *Microbaterium suwonense* and *Glutamicibacter* sp. strains shed light on their metabolic behaviours and adaptability to variable natural conditions. In the study on plant-microbe interactions, it is important to have the possibility to visualize bacteria directly on the host tissues. another objective of the thesis was the use of bacterial transformation techniques, such as electroporation, to introduce fluorescent markers into bacterial strains. This technological innovation enables the in vivo visualization of interactions between bacteria and plants, providing a direct strategy to study how microorganisms behave inside the plant root biological system. This approach could open modern roads in biological and agronomic research. Ultimately, the thesis aims to contribute to promoting more sustainable agrarian practices. The results highlight the significance of compost as a reasonable alternative to chemical fertilizers, recommending that appropriate nutrient and irrigation management can improve plant well-being and resilience to adverse conditions. In summary, the targets of this thesis interlace within a multidisciplinary research system pointed at providing a more profound understanding of plant-microorganism

interactions. Through analyzing plant responses to water stress, optimizing bacterial transformation techniques, and improving sustainable agronomic practices, this research offers significant contributions to tending to modern environmental challenges and promoting more resilient and responsible agriculture.

2. Materials and Methods

2.1 Experimental setup for drought stress

The experimental setup for assessing drought stress in plants was conducted in two phases. Four-week-old plants were cultivated in autoclaved peat pots filled with soil and subjected to different drought conditions by varying the amount of deionized water provided every two days over two weeks. The deionized water was chosen to avoid the introduction of additional salts, which could interfere with the nutrients already supplied by the compost.

The treatments were as follows:

- Control (CTRL): full hydration with deionized water.
- Low Stress (LOW): 35 mL of deionized water.
- High Stress (HIGH): 20 mL of deionized water.

Four soil conditions were tested: one with the addition of compost (SOIL + M33), one with sterilized compost (SOIL + St M33), one with soil and chemical fertilizer (SOIL + NPK) for comparison, and one with only soil (Figure 1).

Dry biomass of the aerial parts and roots of the plants was sampled on Day 1 and Day 14 to assess the impact of the different watering regimes. Additionally, the photosynthetic efficiency, measured by the Fv/Fm parameter using the MultispeQ device, was recorded on Days 1, 7, and 14 to monitor plant stress levels over time.

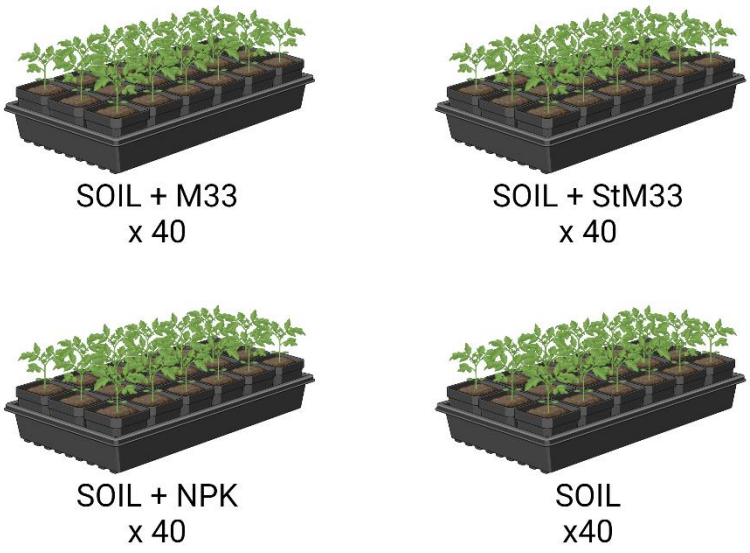

Figure 1. Experimental setup. Created with *BioRender.com*.

2.2 Soil and treatments

The soil used for the experiment consisted of a mixture of 45% agricultural field soil, 45% peat, 5% perlite, and 5% sand. Its chemical and physical properties are shown in Table 1.

Soil characteristic	Unit	Value	Method
pH	pH unit	7,69	DM 13/09/1999 Met III.1
Electric conductibility 1:2	dS/m at 25°C	1,58	DM 13/09/1999 Met IV 1
Very fine gravel (>2mm)	%	<1	DM 13/09/1999 Met II.1
Organic C (Walkley-Black)	g/kg	89	DM 13/09/1999 Met VII.3
Total N	g/kg	2,61	DM 13/09/1999 Met XIV2 + XIV3
C/N		34	DM 13/09/1999 Met XIV2 + XIV3 + VII3

P availability (Olsen)	mg/kg	23	DM 13/09/1999 Met XV3
P availability (P2O5) (Olsen)	mg/kg	53	DM 13/09/1999 Met XV3
Cation-exchange capacity	meq/100g	12	DM 13/09/1999 Met XIII.2
Exchangeable Mg	meq/100g Mg	0,12	DM 13/09/1999 Met XIII.5
Exchangeable Ca	meq/100g Ca	0,76	DM 13/09/1999 Met XIII.5
Exchangeable Na	meq/100g Na	1,3	DM 13/09/1999 Met XIII.5
Exchangeable K	meq/100g K	<0,10	DM 13/09/1999 Met XIII.5
Exchangeable K (K2O)	mg/kg K2O	3,45	DM 13/09/1999 Met XIII.5
Cd	mg/kg Cd	<5,0	DM 13/09/1999 Met XII.1
Cr	mg/kg Cr	42	DM 13/09/1999 Met XII.1
Fe	mg/kg Fe	13274	DM 13/09/1999 Met XII.1
Mn	mg/kg Mn	255	DM 13/09/1999 Met XII.1
Ni	mg/kg Ni	10	DM 13/09/1999 Met XII.1
Pb	mg/kg Pb	14	DM 13/09/1999 Met XII.1
Cu	mg/kg Cu	19	DM 13/09/1999 Met XII.1
Zn	mg/kg Zn	43	DM 13/09/1999 Met XII.1

Table 1. Soil characterization conducted by S.E.S.A S.p.A.

The compost used in this study was provided by S.E.S.A. S.p.A. (Este, Italy) and identified as batch M33.

The compost used in this study had total nitrogen (N) content of 1.8%, a 0.718% content of phosphorus (P), and a 2.072% content of potassium (K). The compost used in this experiment had a moisture content of 47%. Accordingly, the dry matter of compost was proved to have nutrient concentrations as follows: 0.39852 g/kg of nitrogen, 0.15897 g/kg of phosphorus, 0.45874 g/kg of potassium.

To determine the amount of compost to be used in the plant fertilization experiment, we considered the nitrogen requirement for tomato cultivation,

estimated at 150 kg N/ha (personal communication from collaborators in the DAFNAE department, Unipd). This nitrogen amount was used as a basis to calculate the amount of compost, expressed in grams of compost per liter of soil, needed to meet this requirement. The calculation considered both the water content in the compost and the nitrogen release rate in the soil. In fact, only 50% of the nitrogen in the compost is released immediately and becomes available to the plants. Based on these factors, the amount of compost used in the experiment was calculated to be 11 grams of compost per liter of soil.

Other sources of nutrients were meant to balance the nutrient levels and apply the nutrients exactly to the needed concentration. The dibasic ammonium phosphate (DAP) contains 18% nitrogen and 20% phosphorus (as P_2O_5); it was used to fulfil the phosphorus requirement equal to the amount in the compost. From the composted material, the given amount of DAP to supply the equal of P_2O_5 was calculated as shown below:

$$\text{DAP required} = \frac{20\%}{0.15897\ \text{g/kg}} \approx 0.79483\ \text{g/kg}$$

DAP was considered also as a source of N, calculated as following:

$$\text{Nitrogen from DAP} = 0.79483\ \text{g/kg} \times 18\% \approx 0.14307\ \text{g/kg}$$

The rest of nitrogen was provided by urea (CH_4N_2O), a compound that has a N release rate similar to compost. To determine the precise amount of urea needed for the experiment, calculations were performed taking in account the contribution from both diammonium phosphate (DAP) and urea. Given that urea contains 46% nitrogen, we used 0.3110 g per kg of soil.

Potassium was also considered in the formulation of the chemical fertilizer with the same nutrient amount of compost. To provide to plants the same amount of K we used KCl (52% of K in weight), and the quantity of KCl was determined as shown below:

$$\text{KCl required} = \frac{52\%}{0.45874\ \text{g/kg}} \approx 0.88219\ \text{g/kg}$$

2.3 Plant growth

Tomato seeds, *Solanum lycopersicum* L., var. Micro-Tom were surface sterilized for 20 minutes in a 5% sodium hypochlorite solution under gentle shaking using an orbital shaker (~100 rpm). The seeds were then rinsed with sterile deionized water four times for 10-minutes. They were further agitated in sterilized tap water on the orbital shaker for 48 hr at room temperature to obtain a more uniform germination. The surface-sterilized seeds were then plated under aseptic conditions using Murashige & Skoog Basic Medium ½ with 1.5% (w/V) agar into100 mm square Petri dishes. Plates were incubated at 25 °C, with a humidity of 68% and under conditions of a photoperiod alternating between light (16 h) for growth and dark (8 hour). Five days post-germination seedling were transplanted in pots with different substrates: soil, soil mixed with compost M33, soil mixed with compost M33 sterilized, and soil with chemical fertilizer. Plants were moved to peat pots a week before starting the drought stress treatment, so that they could get established in the new pot.

2.4 Maximum photosynthetic efficiency

Photosynthetic efficiency was measured using a MultispeQ from PhotosynQ, a device recording chlorophyll fluorescence at the level of PSII. Efficiency measures were recorded on days 1, 7, and 14 of the experiment.

The instrument first recorded the minimum fluorescence, Fo, and then turned on a short saturating light pulse, closing all available reaction centers and measuring the maximum fluorescence, Fm. Variable fluorescence, Fv, was calculated as follows:

$$F_v = F_m - F_o$$

The Fv/Fm ratio, representing the maximum efficiency of photosynthesis, was derived from these measurements. This ratio is a key indicator of plant health and response to environmental stress. The Fv/Fm ratio is calculated as:

$$\text{Fv/Fm ratio} = \frac{F_v}{F_m}$$

A Fv/Fm ratio ranging from 0.79 to 0.84 is considered optimal for many plant species, with values approaching 1 indicating maximum photosynthetic efficiency. Lower values suggest stress or damage to the plant (Maxwell and Johnson, 2000).

Statistical analyses were performed using the Kruskal-Wallis test to detect significant differences in photosynthetic efficiency between treatments. Pairwise comparisons were then conducted using Dunn's test to identify specific differences among groups.

2.5 Leaves biomass

Dried leaves from plants of the various treatments were weighed for dry biomass after five days in an oven set at 60°C using analytical balance. We evaluated differences in dry biomass between the treatments with a Kruskal-Wallis rank sum test. The test for pairwise comparisons between groups was Dunn's test and p-values were adjusted with the Benjamini-Hochberg (BH) method to control the false discovery rate.

2.6 Water potential

Water potential of the plants was measured using a Schölander pressure chamber. In principle, this device measures the amount of pressure that pushes water out of plant tissues, where the pressure created is a measure of the water potential of the plant.

Procedure:

- Sample Collection and Preparation:
  - Leaf samples were collected on Day 1 and Day 14 of the experiment.
  - Leaves were immediately covered with parafilm to prevent loss from the samples.
- Device Calibration:
  - Calibration of the Schölander pressure bomb according to the instructions.
  - Setting the pressure gauge to zero.
- Preparing Leaves:
  - A leaflet was mounted in the specimen holder of the chamber.
  - Avoiding air bubbles or extra water drops with much care since they could interfere with the measurement.

- Measurement Technique:
    - The chamber was sealed, and pressure was gradually applied as water began to exude from the cut surface of the leaf
    - The pressure applied at this point was recorded and the resulting water potential was expressed as a negative value.

The recorded data for water potential on Day 1 and Day 14 were analysed to report differences using the Kruskal-Wallis test among the treatment groups. The pairwise comparisons were performed by a Dunn test to indicate which groups are significantly different. This will allow for the determination of how the different experimental conditions impacted the plant's water potential over time.

2.7 Bacterial growth curve

Bacterial growth curve experiment was conducted for the bacterial strains *M. suwonense* and *Glutamicibacter* sp. Growth curves are essential tools as they provide insights into the growth kinetics of bacteria, enabling an estimation of the bacterial cell count in a batch culture grown under consistent conditions by measuring its optical density at 600 nm (OD600). OD600 readings were collected using a UV-vis spectrophotometer (Cary 60 UV-Vis, Agilent).

The growth curve was obtained for each strain with the following procedure:

- Collect bacterial strains from -80°C stocks with a sterile inoculation loop and inoculate them in 5 mL of liquid PCA ½. Incubate this pre-inoculum overnight at 28°C under agitation on the orbital shaker.
- Inoculate 100 µL of pre-inoculum in 10 mL of liquid PCA ½.
- Measure the OD of the culture (OD 0).
- Incubate the culture at 28°C under agitation on the orbital shaker and measure the OD after 4 hours (OD 4), 8 hours (OD 8), 24 hours (OD 24), 28 hours (OD 28), 32 hours (OD32), 48 hours (OD48) and 52 hours (OD52).
- Simultaneously with each measurement, collect 0,5 mL of the culture to perform plate counts and assess the number of bacterial cells in the culture at each time point. Directly plate 100 µL of the culture with the spread plate method on PCA agar. Perform a serial dilution of the

culture with liquid PCA ½ to make the colony counting possible: 1:2, 1:100, 1:1000, 1:10000, 1:100000. Plate 100 µL of each dilution with the spread plate method on PCA agar and incubate at 28°C for 24 hours.

- After this period, count the colonies on the plates and multiply their number by the appropriate dilution factor to calculate the CFU/mL in the original sample.
- Calculate a bacterial growth curve for each strain using OD and plate count data.

Each growth curve was visually examined to identify its linear phase, and the equation for this segment was calculated. This equation was then used in subsequent experiments to determine the log (CFU/mL) of the bacterial cultures by measuring their OD600.

2.8 Bacterial transformation by electroporation

The following media have been prepared in advance:

- LB (Luria-Bertani) Medium (both solid and liquid media).
- NB (Nutrient Broth) Medium (both solid and liquid media).
- Growth Medium
- Electroporation Medium
- Recovery Medium

The following Procedure has been followed to obtain transformed cells:

- Preparation of Bacterial Cultures:
  - bacteria were streaked from -80°C stock onto LB solid medium and incubate at 28°C overnight.
  - a colony was selected and grow it in 5 mL of growth medium overnight at 28°C to an OD of 0.85-0.95.
- Bacterial Preparation:
  - bacterial cultures were chilled on ice for 10 minutes, then centrifuged at 4°C, 3200 rpm for 5 minutes.
  - pellets were resuspended in 1 mL of electroporation medium.

- bacteria were washed 3 times in the electroporation medium, and finally resuspended in 100 µL of electroporation medium (target concentration approximately 1-1.3 x 10^10 CFU/mL).

- Electroporation:
  - for the transformation 2 µL of the plasmid of interest were added to aliquots of 60 µL of bacteria.
  - the mixture was transferred into the pre-chilled cuvette and incubated on ice for 10 minutes.
  - the cold cuvettes with bacteria were inserted into the electroporation device (BioRad MicroPulser), and pulsed with 2.5 kV (Program Ec2), the time constant resulted to be approximately 4.5–5 ms.
  - 1 mL of recovery medium (at room temperature) was added immediately after the pulse into the cuvette, and incubated at 28°C with gentle agitation for 3 hours.
- Plating:
  - after recovery, bacteria were Plated on LB/NB solid media containing the appropriate antibiotic and incubate at 28°C.

Composition of the media used in this experiment are the following:

- Growth Medium: Sorbitol 0.5 M in LB medium
- Electroporation Medium:
  - Sorbitol 0.5 M
  - Mannitol 0.5 M
  - Trehalose dihydrate 0.5 M
  - Glycerol 10%
- Recovery Medium:
  - Sorbitol 0.5 M
  - Mannitol 0.38 M
  - in LB medium

2.9 Growth medium

The Murashige & Skoog (MS) medium is a widely used culture growth medium for plant tissues (Murashige & Skoog, 1962). It includes a balanced combination of inorganic salts, vitamins, organic compounds, and plant growth regulators. For the experiments, we used half-strength Murashige & Skoog medium (MS½), which contains half the concentration of the nutrients compared to MS.

Plate Count Agar (PCA) is a type of growth medium commonly used to determine the total number of viable bacteria or yeast cells in a sample (Buchbinder, 1951). It is not a selective medium.

Ingredients:

- Yeast extract
- Tryptone
- Glucose

Luria-Bertani (LB) broth is a versatile growth medium commonly used to grow a wide range of bacterial species (Bertani, 1951). It is a nutrient-rich medium, supplying amino acids, peptides, and other essential nutrients necessary for bacterial growth.

Ingredients:

- Tryptone
- NaCl
- Yeast Extract
- Agar (for solid medium)

Nutrient Broth (NB) is a widely used growth medium formulated to support the growth of a broad spectrum of microorganisms (Low, 2013). It is a nutrient-rich medium providing essential components such as beef extract, peptone, and yeast extract to promote the growth of various bacterial species.

Ingredients:

- Beef Extract

- Peptone
- Yeast Extract
- NaCl
- Agar (for solid medium)

2.10 Statistical analysis

Data obtained from the experiments were analyzed through RStudio, an Integrated Development Environment for the programming language R. The package used to create boxplots was *ggpubr*.

3. Results and Discussion

3.1 Drought stress

3.1.1 Preliminary experiment 1

In the first series of experiments, we performed preliminary experiments to set up the conditions and optimize the analyses of the response of plants to drought stress conditions. Tomato plants, grown in sterilised peat pots, were three weeks old when the experiment was set up (Hakeem & Akthar, 2016). Plants were grown in a mix of soil, soil added with compost, soil added with a chemical fertilizer (NPK), and soil added with sterilized compost. Initially, we defined four levels of drought stress: the control group (CTRL) received 25 mL of deionised water every two days, the low-stress (LOW), moderate-stress (MILD), and high-stress (HIGH) groups received 12.5 mL, 6.5 mL and 0 mL of water, respectively. After two weeks of treatment, we sampled the aerial parts and roots of the plants to measure the dry biomass.

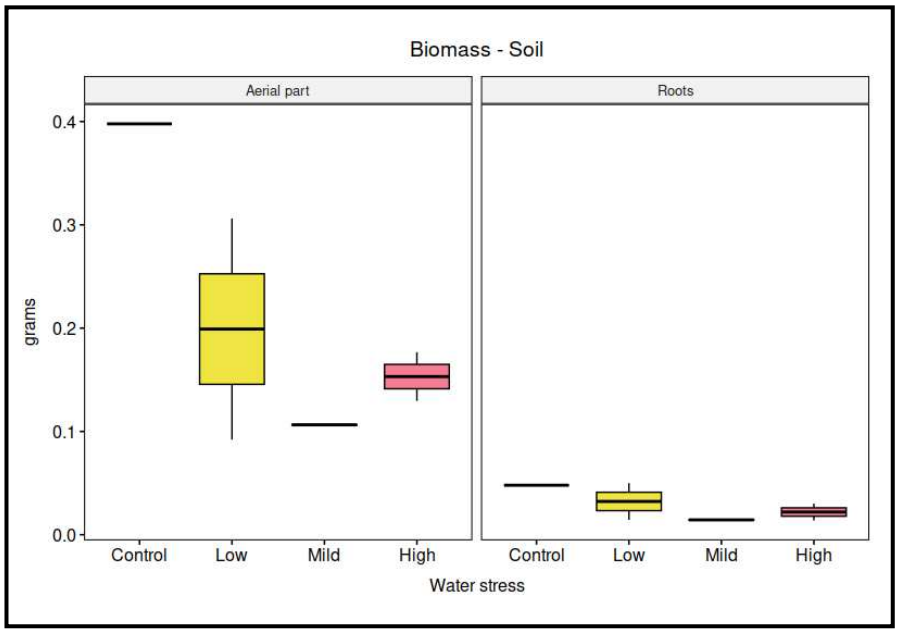

Figure 2. Boxplot representing the distributions of the biomass of the aerial part and of the roots of the plants grown in soil without compost for the preliminary experiment 1

The first results, as it's show in Figure 2, indicated that, even for the control group, the amounts of water provided were insufficient and created stress also in the control plants. This observation couldn't allow us to make meaningful statistical analyses, as the number of replications was too low-only three replications per condition- but permitted us to identify the correct range of water amount for each set of plants. Therefore, in the next experimental setup

we will add compost, sterile compost and chemical fertilizer to the soil, thus creating three different conditions for further studying soil-plant interactions (FAO, 2022; Ullah, 2021) and adjusting water stress for more reliable data.

3.1.2 Preliminary experiment 2

In the second series of experiments, optimized water stress conditions were applied: the volume of the water applied was increased to 75 mL for the control, 35 mL for the low-stress, and 20 mL for the moderate-stress treatments. The irrigation protocol has been repeated every two day for two weeks. For this reason, we sampled the aerial parts and the roots of the plants, recording the dry biomass on days 1 and 14, measuring photosynthetic efficiency via the Fv/Fm parameter by using the instrument MultispeQ on days 1, 7, and 14.

The results (Figure 3) related to photosynthetic efficiency showed that when the plants are grown with compost, there is no significant difference among all different levels of stress ($p > 0.05$), which means that none of them is overstressed. On the other hand, in the case of sterile compost when plants were under stress for 7 days (Figure 3 B), the moderate stress level showed significantly lower photosynthetic efficiency than in the case of the control and low stress groups ($p = 0.002$).

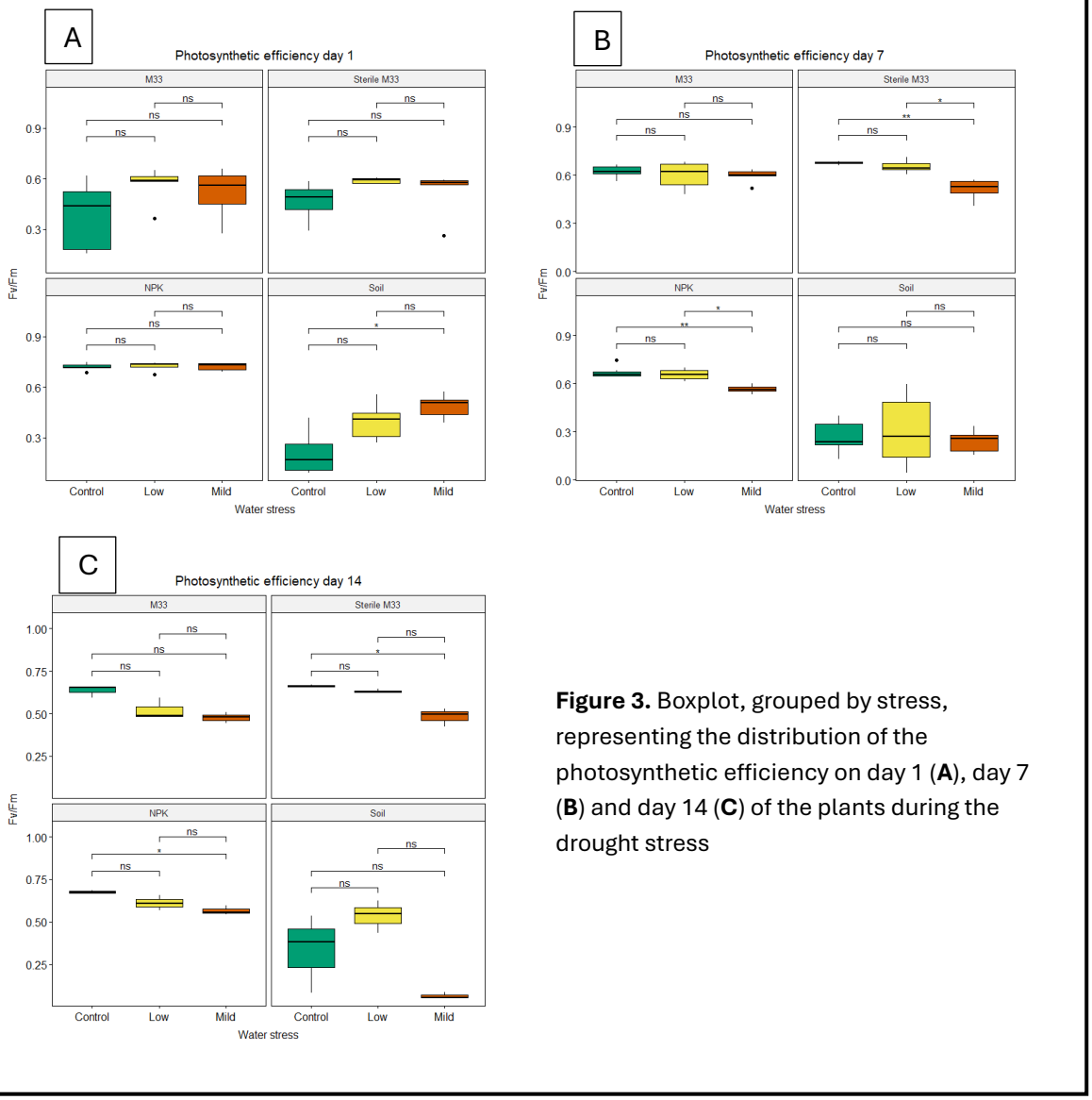

Figure 3. Boxplot, grouped by stress, representing the distribution of the photosynthetic efficiency on day 1 (**A**), day 7 (**B**) and day 14 (**C**) of the plants during the drought stress

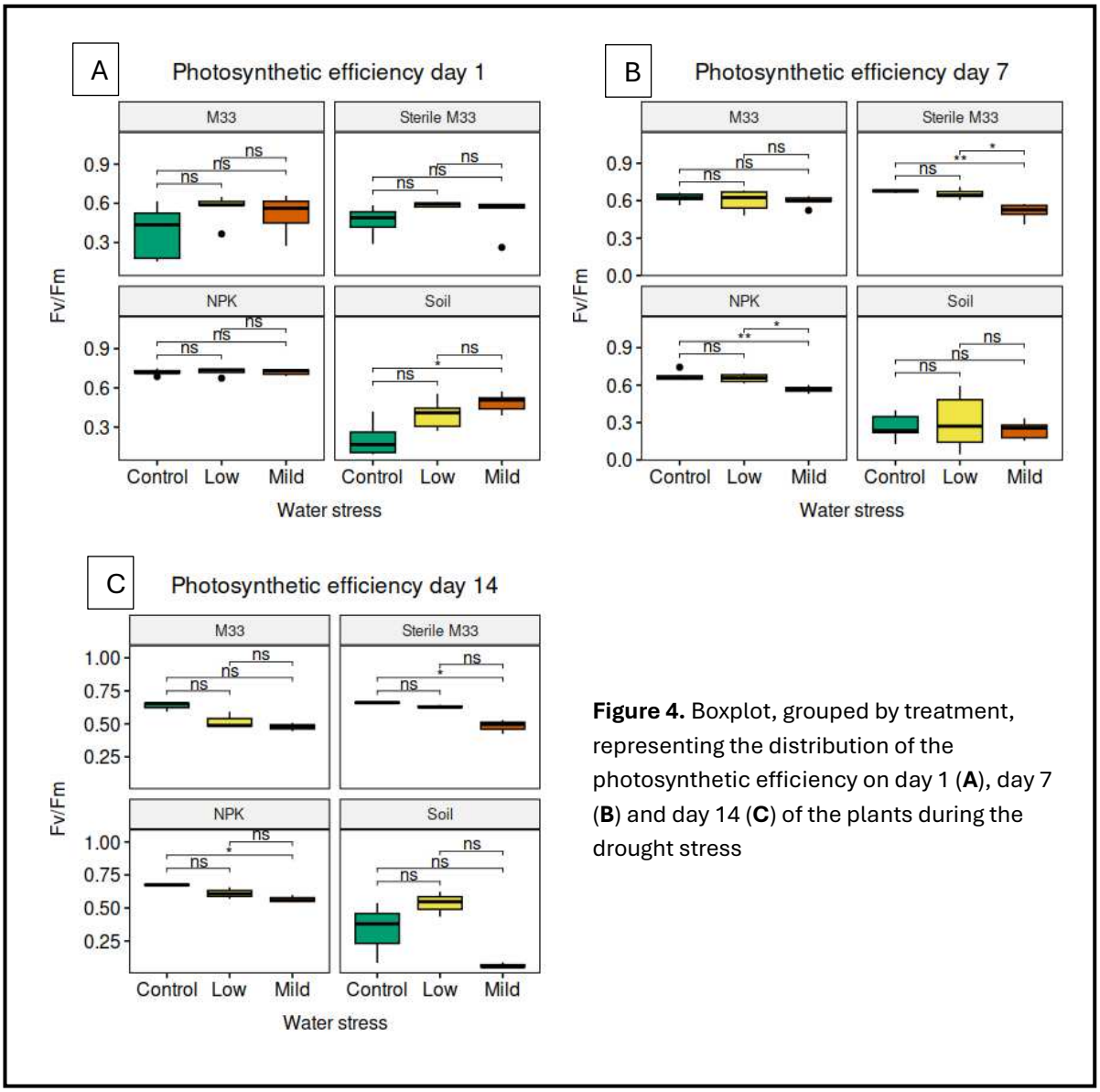

Figure 4. Boxplot, grouped by treatment, representing the distribution of the photosynthetic efficiency on day 1 (**A**), day 7 (**B**) and day 14 (**C**) of the plants during the drought stress

This could mean that due to the lack of compost microbiota, a more vulnerable and photosynthetically less effective plant was the result (Compant, 2024 and Cataldo et al., 2022). Besides, plants that were treated with chemical fertiliser were more sensitive to stress (Figure 4) as compared to the plants grown using compost ($p = 0.017$), which shows the importance of soil microbiota for plant resilience (FAO, 2022).

No significant difference in the dry biomass was recorded among the various treatments ($p > 0.05$), both for the aerial part and for the roots (Figure 5 and 6). However, we can recognise a trend that could indicate possible biomass loss after a longer time, beyond the two-week period of the experiment. Large biological variability among plants could have reduced the reliability of the conclusions, suggesting the use of a higher number of experimental plants.

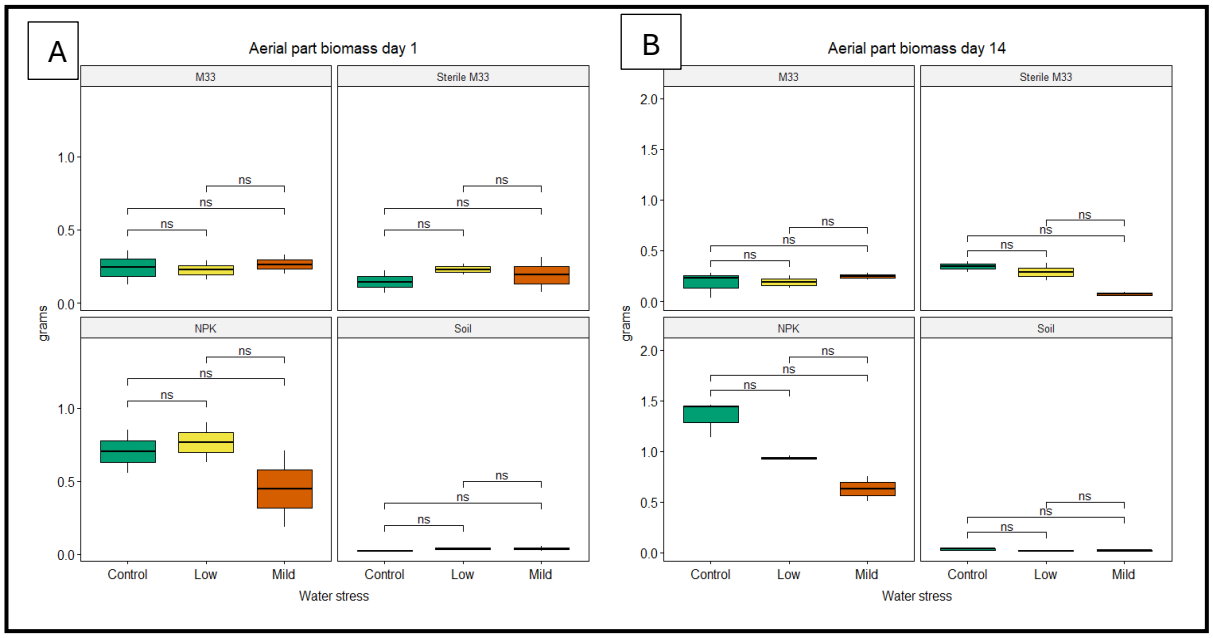

Figure 5. Boxplot representing the distributions of the biomass of the aerial part on day 1(**A**) and day 14 (**B**)

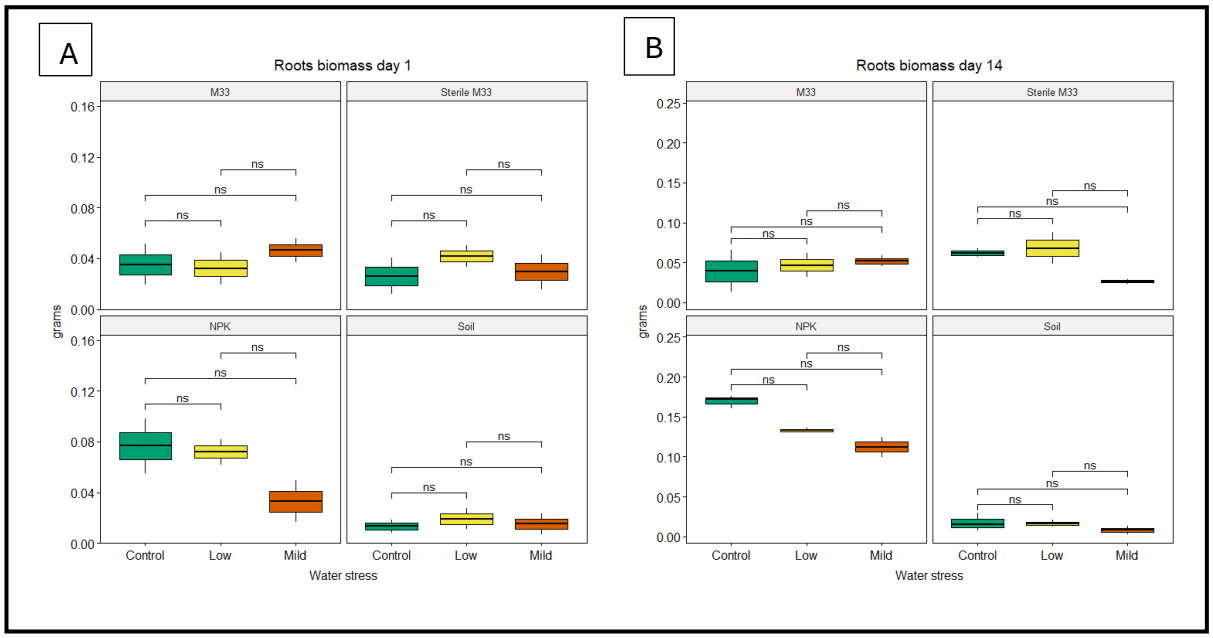

Figure 6. Boxplot representing the distributions of the biomass of the roots on day 1(**A**) and day 14 (**B**)

3.1.3 Experiment 1

Driven by the data collected in the preliminary experiment, we set up a new round of trials, in which we maintained the same drought stress conditions, using 75 mL of water for the control group, 35 mL for the reduced stress group and 20 mL for the moderate stress group. We sampled the aerial parts and

roots of the plants to measure the dry biomass and performed photosynthetic efficiency measurements using the Fv/Fm parameter with the MultispeQ instrument on days 1, 7, and 14.

The photosynthetic efficiency measurements revealed that, on day 1, before any stress was applied, all the plants showed a comparable photosynthesis level and didn't show any sign of stress (Figure 7 and 8). After 7 days, the plants grown with compost did not show any sign of stress in any substrate (Figure 7 B and 8 B); however, plants grown with sterile compost showed a level of photosynthetic efficiency significantly lower in the mild stress ($p = 0.001$), compared with the control plants. This trend can also be hypothesized for the plants fertilised with NPK, even if the result is not statistically significant and clearly evident ($p = 0.345$). At 14 days, all the plants subjected to mild stress resulted in lower Fv/Fm values (Figure 7 C and 8 C); the plants subjected to low stress showed a mixed and apparently contradictory behaviour: plants with compost showed a significant level of stress ($p = 0.024$), while plants grown in sterile compost and NPK fertilizer did not show stress when compared with the controls ($p = 0.165$ and $p = 0.120$, respectively): this result could suggest that in the long term, the plants with compost performed a little worse than the plants with sterile compost and NPK, where the compost microbiota is not present. This could be explained in different dynamics of nutrient availability and consumption. In any case, a prolonged stress time like 14 days is not very representative of agricultural cultivation.

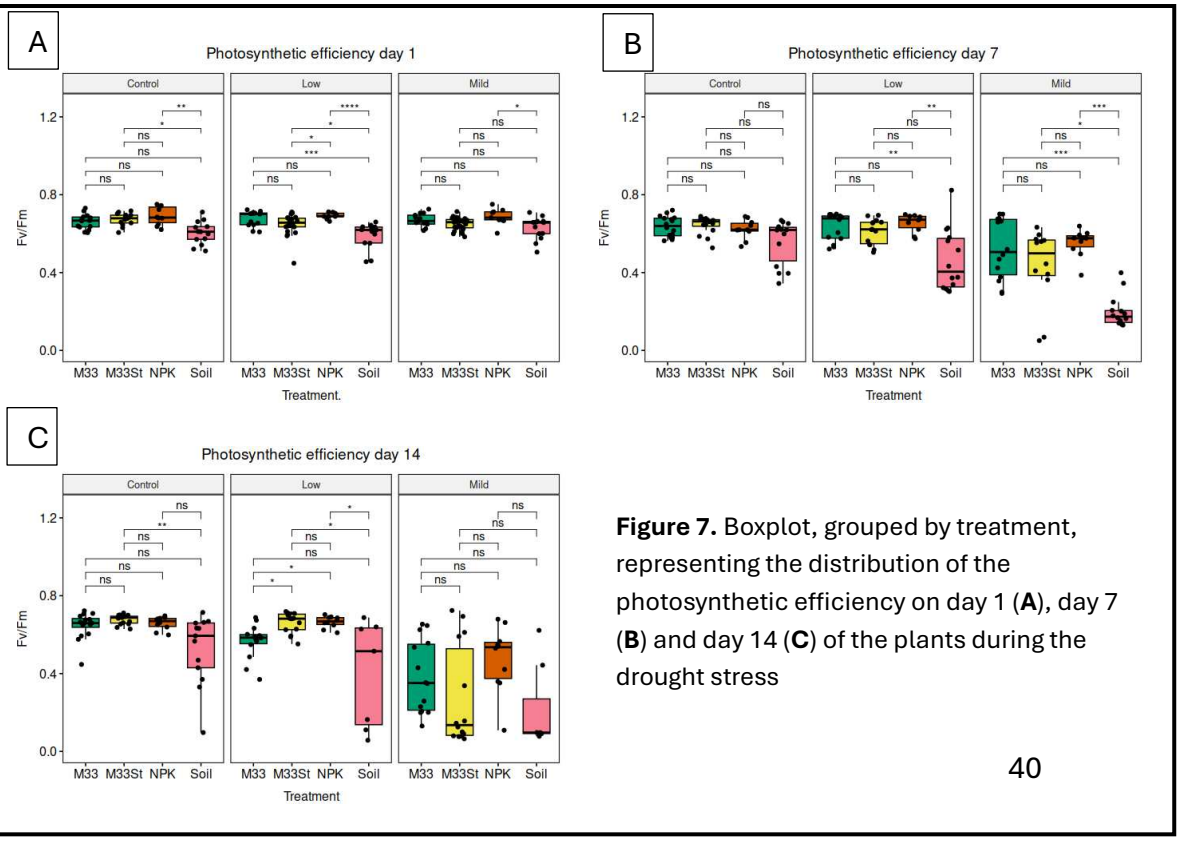

Figure 7. Boxplot, grouped by treatment, representing the distribution of the photosynthetic efficiency on day 1 (**A**), day 7 (**B**) and day 14 (**C**) of the plants during the drought stress

Although no significant differences in dry biomass (Figure 9) emerged between the treatments ($p > 0.05$), the overall results showed that the plants had a good nutrient supply and conditions in the climate chamber were optimal.

The plants' physiological reaction to these growth conditions is highlighted by the decrease in biomass seen under stress in sterile compost and NPK-nutriented substrates (Figure 9). This trend might suggest that, despite offering vital nutrients, the lack of an active microbiome (in the case of sterile compost) or a complex organic matrix (in the case of NPK) reduces the ability of plants to resist environmental stress. The finding leads to reflecting on the significance of the interaction between nutrients and the soil microbiota in promoting plant development and reducing the impact of abiotic stresses.

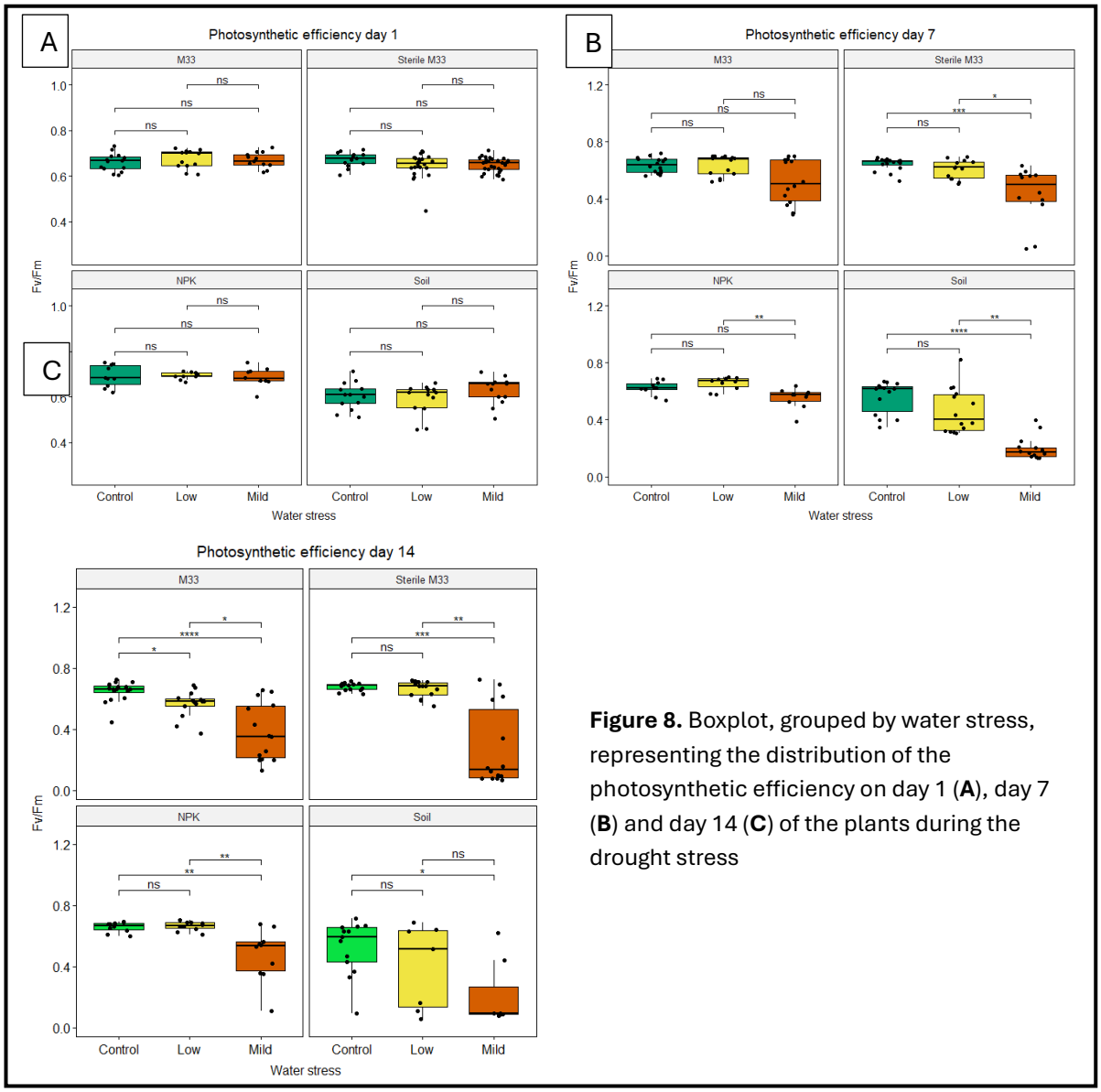

Figure 8. Boxplot, grouped by water stress, representing the distribution of the photosynthetic efficiency on day 1 (**A**), day 7 (**B**) and day 14 (**C**) of the plants during the drought stress

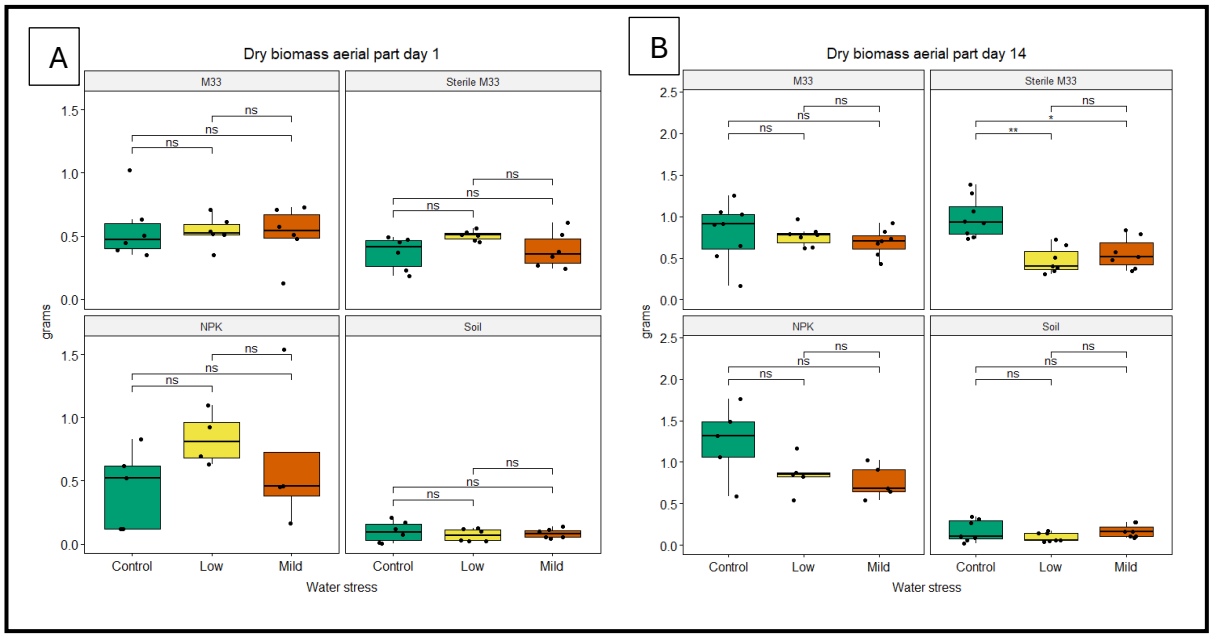

Figure 9. Boxplot representing the distributions of the biomass of the aerial part on day 1(**A**) and day 14 (**B**)

In addition, plants grown in composted soil showed increased resistance to water stress, especially in the early stages (Figure 7 and 8). This suggests that the micro-organisms in the compost may help plants cope with stress by improving water uptake and providing essential nutrients. This observation supports the idea that although plants with NPK and sterilized compost have good nutritional levels, the lack of an active microbiome could compromise their ability to cope with environmental challenges in the long term.

3.1.4 Experiment 2

The previous experiment was replicated to have a higher number of replicates and confirm the results and trends observed in the first trial. The experimental groups remained unchanged, with the control group receiving 75 mL of water, the reduced stress group 35 mL and the moderate stress group 20 mL, for a period of two weeks. As in the previous phases, we sampled the aerial parts and roots of the plants, measuring the dry biomass, and made detailed measurements of the water potential using the Schölander chamber (Arora & Bouizgarne, 2022).

The results on photosynthetic efficiency showed that at higher stress levels the compost seemed to perform better, demonstrating a significant difference compared to the other treatments ($p < 0.05$).

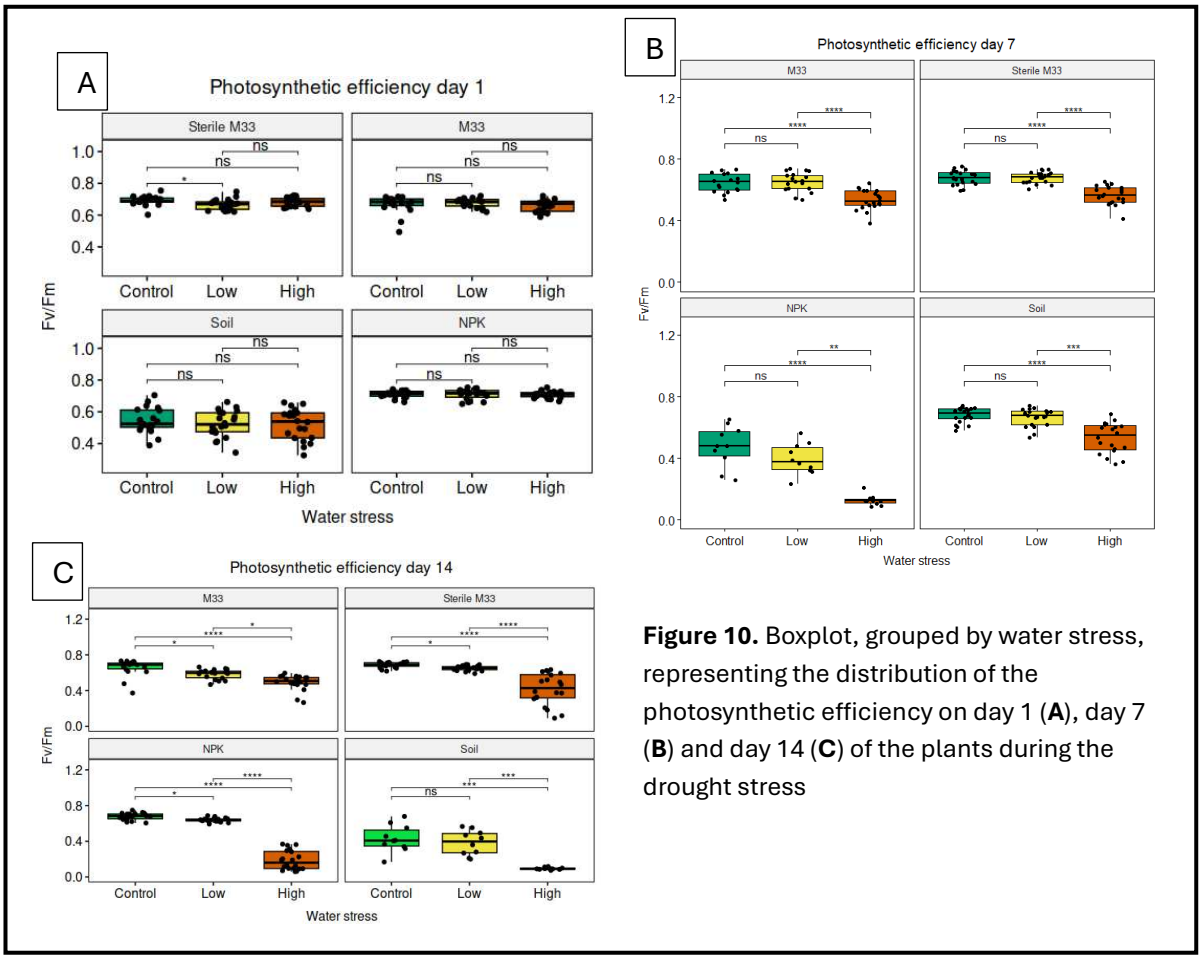

Figure 10. Boxplot, grouped by water stress, representing the distribution of the photosynthetic efficiency on day 1 (**A**), day 7 (**B**) and day 14 (**C**) of the plants during the drought stress

However, the effect of compost was not significantly different from that of sterile compost (p = 0.469), raising questions if it is a response to stress per se (Soria, 2022; Lamaizi, 2023). Indeed, despite the generally good performance of compost, we received an anomaly in the data (Figure 10 B): a slight increase in photosynthetic efficiency on no compost samples at day 7 (p = 0.36), which we are unable to explain. From this, on day 14, there was a decline in photosynthetic efficiency (p = 0.0753), showing that the results did not hold consistency (Figure 10 and 11)

A comparison of data by treatment type (Figure 11) reveals that there is considerable variation in photosynthetic efficiency between controls and high stress conditions (p = 0.511 for SOIL CTRL - HIGH and p = 0.345 for M33 CTRL - HIGH), hence indicating that the type of treatment significantly influences the response of the plants (Alsadon, 2024; Lamaizi, 2023; Soussani, 2023).

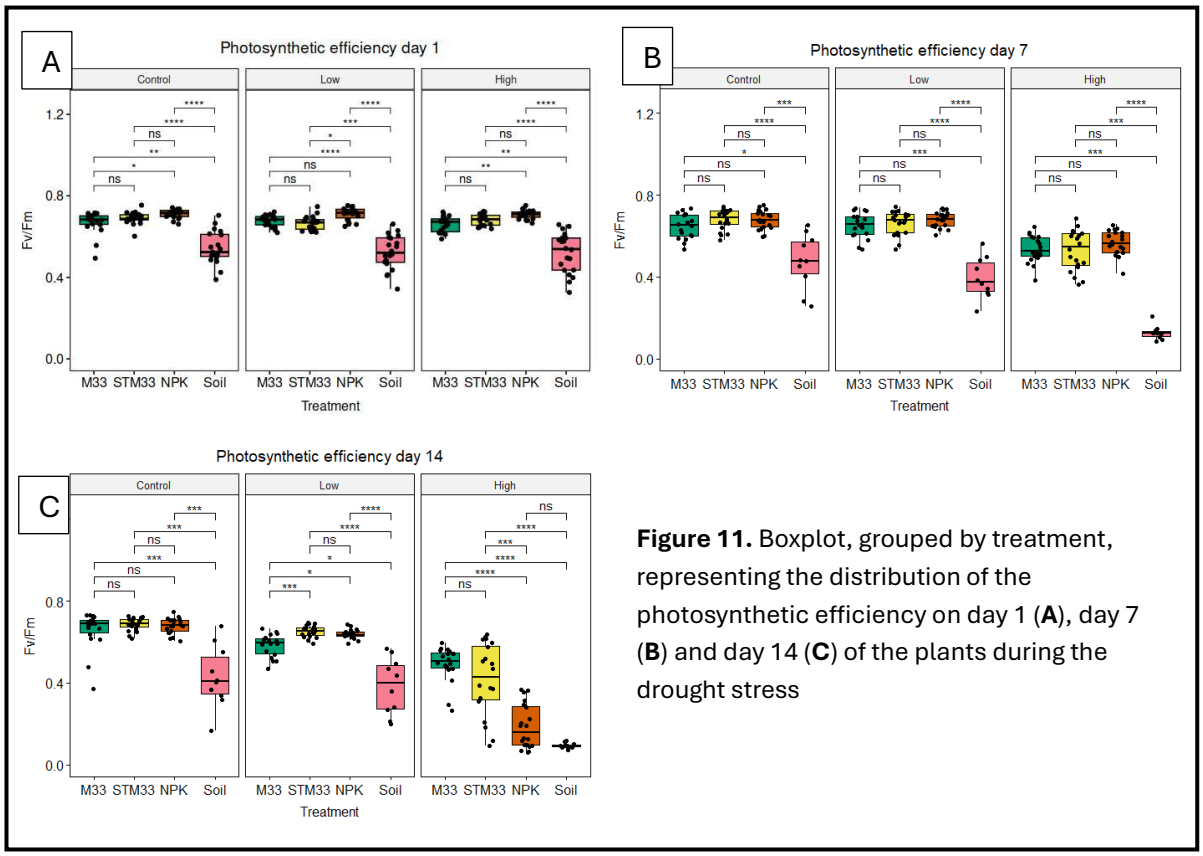

Figure 11. Boxplot, grouped by treatment, representing the distribution of the photosynthetic efficiency on day 1 (**A**), day 7 (**B**) and day 14 (**C**) of the plants during the drought stress

However, it is crucial to emphasise that there were no significant differences in dry biomass ($p > 0.05$), indicating that the plants received adequate nutrition and that conditions in the climate chamber were optimal (Figure 12 and 13).

It is important to note that despite the differences in photosynthetic efficiency, plant biomass did not always show significant differences between treatments. This could be due to biological variability between plants or the fact that the experiments were conducted over a relatively short period of time.

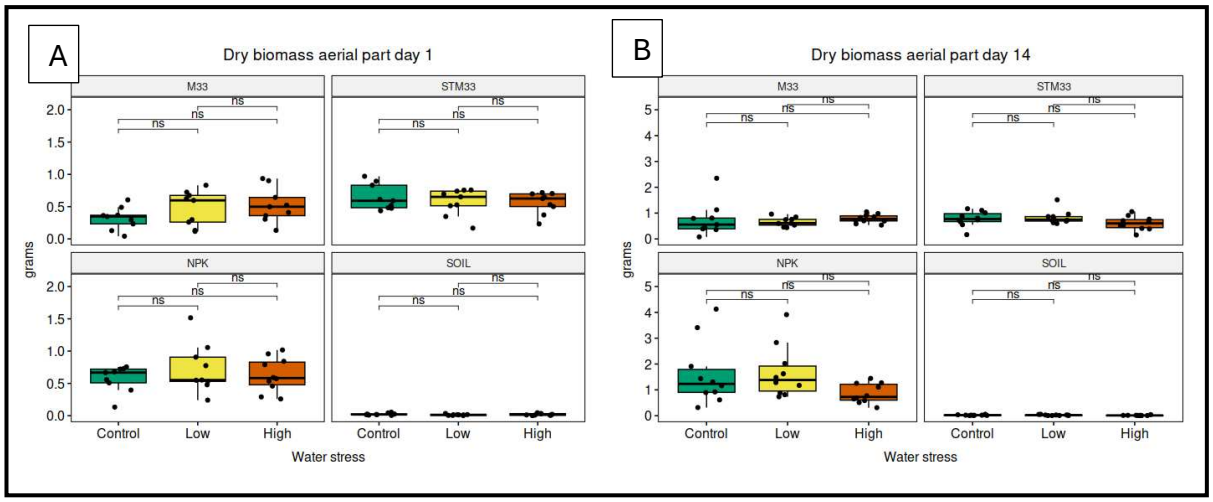

Figure 12. Boxplot, grouped by water stress, representing the distributions of the biomass of the aerial part on day 1(**A**) and day 14 (**B**)

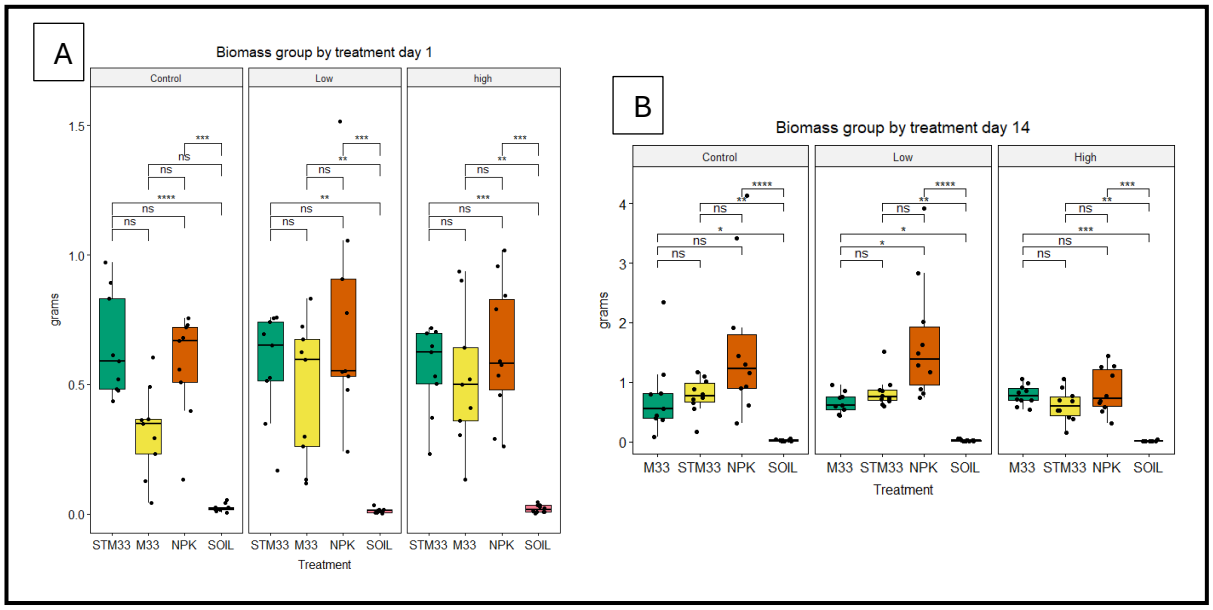

Figure 13. Boxplot, grouped by treatment, representing the distributions of the biomass of the aerial part on day 1(**A**) and day 14 (**B**)

Finally, measurements of water potential (Figure 14) also showed no significant differences ($p > 0.05$), which could be an indication that stress levels were relatively low and that the plants maintained favourable conditions. Clearly, there are opportunities to improve the measurement techniques so that more accurate data can be acquired for this type of experimental planting.

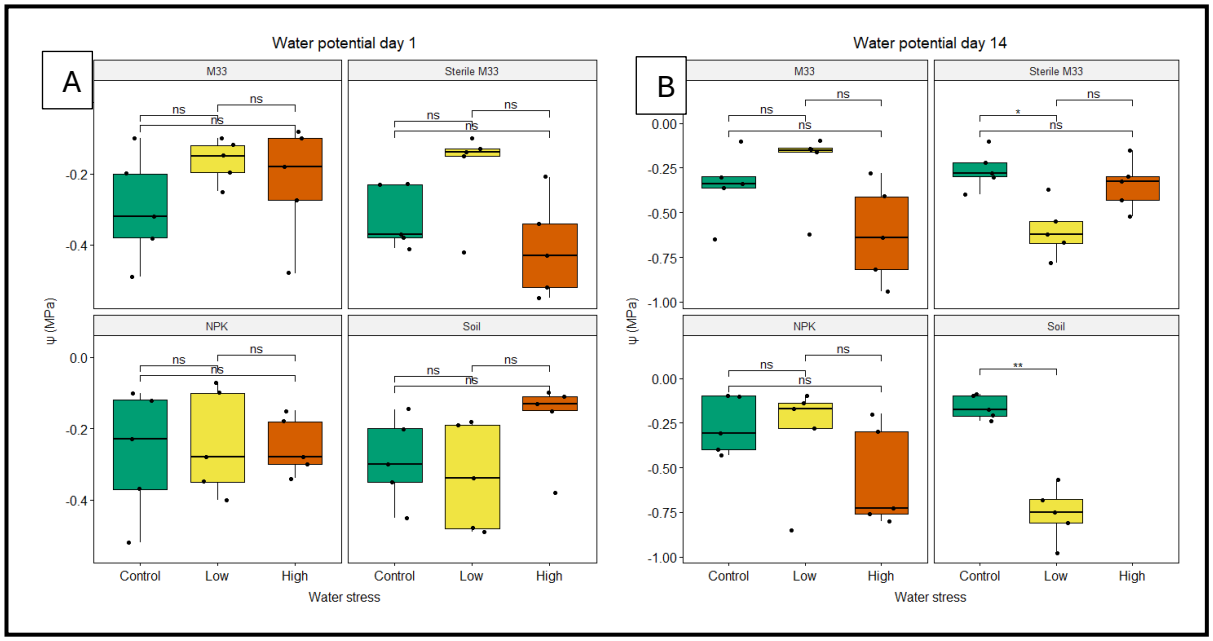

Figure 14. Boxplot representing the distributions of the water potental of the plants on day 1(**A**) and day 14 (**B**)

However, as the water stress continued, the differences between the soils became less apparent. In the second experiment, all plants showed a significant decrease in photosynthetic efficiency after 14 days of stress, regardless of soil type. This may indicate that the prolonged stress was too severe for all plants, even those grown on compost.

Overall, the results suggest that compost can improve the resistance of tomato plants to water stress, at least in the early stages of stress. However, further research is needed to confirm these results and to identify the specific mechanisms by which compost helps plants to cope with stress.

Taken together, these results offer a fundamental insight into the interactions between plants and water stress conditions, providing valuable insights into the sustainable management of water resources in agriculture (Sayyed, 2019).

3.2 Growth curves

Compost has been considered for many years as a soil fertilizer, for its amount of nutrients and organic matter. Recently, compost has been found to have its own microbiota that has been investigated. Many bacterial strains have been isolated. In this thesis, we describe the growth kinetics of two strains: *Glutamicibacter* sp. and *Microbacterium suwonense*.

From the results we can observe a differential behaviour between the two. *M. suwonense* grew faster to reach the exponential growth phase before Glut did (Figures 15 and 16), suggesting greater efficiency in metabolism under culture conditions (Kamakura, 2023). Therefore, it is likely that *M. suwonense* had more efficient enzymes or a faster metabolic regulation to adapt faster to the initial conditions of this culture medium. The fact that it initiates cell division so rapidly may indicate that it uses its resources in a much more efficient way, which is an aspect for further investigation (Fernandez-Martinez, 2024; Mira, 2022).

On the other hand, *Glutamicibacter* sp. evidenced a much lower growth rate, possibly due to higher metabolic complexity or slower biochemical control, or suboptimal conditions in this medium. However, *Glutamicibacter* sp. has a longer stationary phase compared with *M. suwonense*-a survival strategy that allows it to maintain a population stable for much longer, even in an adverse environmental situation.

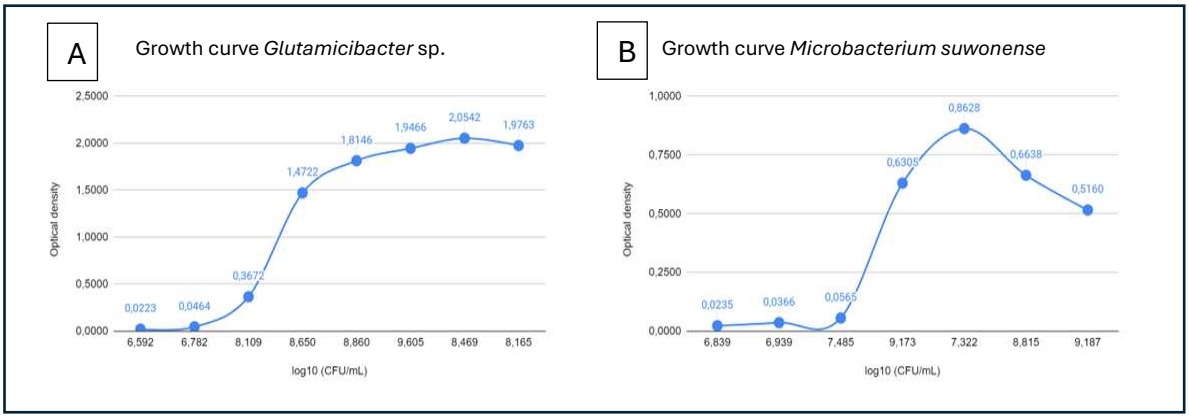

Figure 15. Graph correlating the Log(CFU/mL) and the absorbance for *Glutamicibacter* sp. (**A**) and *M. suwonense* (**B**)

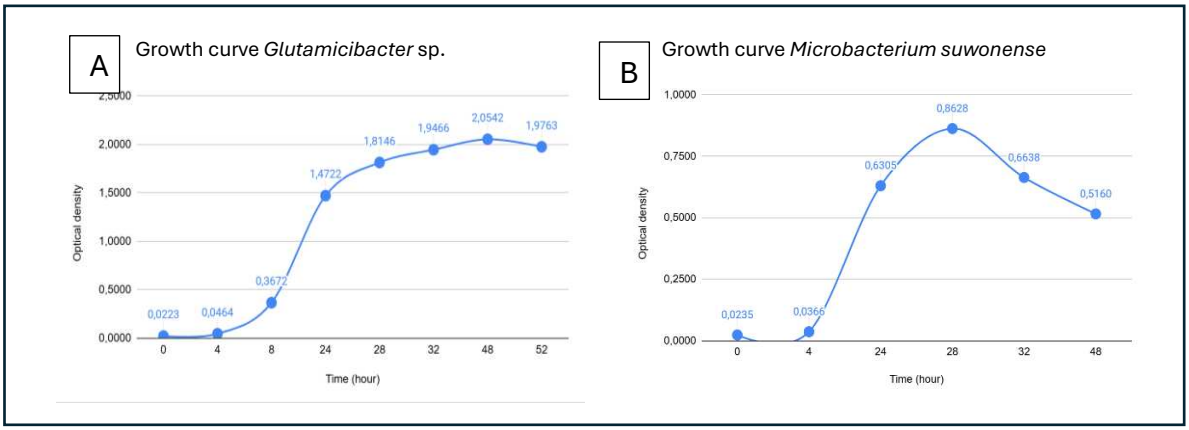

Figure 16. Graph correlating the time (hour) and the absorbance for *Glutamicibacter* sp. (**A**) and *M. suwonense* (**B**)

The prolongation of the stationary phase in *Glutamicibacter* sp. could be advantageous compared to *M. suwonense* which, although growing faster, rapidly depletes resources and has a shorter stationary phase, suggesting a lower capacity to resist prolonged environmental stress, such as the accumulation of toxic metabolites (Kamakura, 2023; Rogers, 2022).

In conclusion, the analysis of growth curves revealed differences not only in growth rates, but also in the survival strategies adopted by the two strains. While *M. suwonense* favours rapid growth, *Glutamicibacter* sp. adopts a more conservative strategy, prolonging the stationary phase. These results offer a more complete understanding of the physiological dynamics of bacterial strains and may be useful for future environmental, industrial or biotechnological applications. Further studies should explore the molecular mechanisms underlying these differences and the role of environmental factors on the cell cycle of the two strains. In previous experiments these strains have shown biochemical activities with a plant growth promotion effect: the study of the dynamic of growth can give us important information also on the interactions that the bacteria could establish with the plant.

The graphs of Figure 15 were also processed to correlate optical density with colony forming units, CFU, to provide a quantitative estimate of microbial growth. This correlation allows an indirect assessment of biomass and provides an additional method of monitoring cell proliferation over time.

The way bacteria interact with plant roots can be greatly influenced by their rate of growth. Bacteria with high growth potential, or those that reproduce quickly, can colonize the rhizosphere more effectively than slow-growing bacteria (López, 2023). The reason for this is that bacteria that grow quickly can take advantage of the nutrients in the root exudates that plants release, which increases their number in the rhizosphere and strengthens their bond with the roots.

3.3 Bacterial genetic transformation

In the study of plant-microbe interactions the localization of the bacteria on or within plant tissues is crucial to fully understand the location and the mechanisms involved in this relationship. There are different approaches to visualize bacteria on and in plant tissues: staining with bacterial specific stains, FISH, and the use of bacterial strains modified to produce fluorescent proteins. This approach includes the genetic transformation of the studied strains with plasmids that harbour genes for fluorescent proteins. In our

research work, a bacterial transformation experiment was conducted on some strains isolated from compost, in order to detect in the future if and where they can interact with plant tissues. For the genetic transformation, we choose the method of electroporation, which is one of the most effective and widely used techniques allowing the introduction of plasmids into bacterial cells (Ozyigit, 2020). The process is crucial in many aspects of biotechnology, since it allows for the transfer of interest genes to host cells, thus enabling the expression of specific traits that might be monitored and studied later on (Cadoret, 2014). The main objective of the transformation, in our case, was to provide specific fluorescent markers to the bacteria strains that would be useful in investigating and understanding the interaction in vivo between bacteria and plants.

For this, we chose well-characterised plasmids, which included pUT-RFP3, sacB-GFP, pUT-GFP3, and mCherry, and combined these with bacterial strains such as *Kocuria rhizophila*, *Bacillus licheniformis,* and *Bacillus subtilis*.

The procedure of electroporation had to be carried out with utmost care because all parameters that set up an electroporator affect the outcome of transformation significantly (Drury, 1994; Mohamadzadeh, 2024). The voltage, pulse duration, and number of pulses were some set parameters on which the setting up of an electroporator was done. Each of these was adjusted according to the bacterial strain used along with the plasmid. For instance, the voltages applied in the various combinations of the plasmids with *Kocuria rhizophila* were 2.5 kV and 3 kV, while for *Bacillus licheniformis*, the voltage applied was 2 kV. This, therefore, calls for careful consideration in the calibration of experimental conditions for any ultimate transformation success.

One of the critical steps involved in our process included the use of glycine, a chemical agent that increases the permeability of cell walls. This becomes important because it would not be possible to easily penetrate plasmid DNA through electroporation in bacterial cells without this agent. Besides, glycine was added in different concentrations, where the concentration of 0.75% was found to be the most effective in the transformation process, where various conditions of transformation were tried. This method improved the efficiency of the process by showing that optimization of cell preparation may play an important role in the realization of high transformation rates.

Strain	Plasmid	Glycine	Voltage (kV)	Recovery	Transformation
K. rhizophila	pUT-RFP3	/	2,5	3h	yes
	pUT-RFP3	/	2,5	3h	yes
	pUT-GFP3	/	3	3h	yes
	sacB-GFP	/	2	ON	/
	sacB-GFP (400ng)	0,75%	2,5	ON	/
	mCherry (400ng)	0,75%	2,5	ON	yes
B. subtilis	pUT-RFP3 (236 ng)	/	2,5	3h	/
	pUT-RFP3	/	2,5	3h	/
	pUT-GFP3	0,75%	3	3h	/
	pUT-GFP3	0,75%	2	ON	/
	pUT-GFP3	0,75%	2	ON	/
	mCherry (500ng)	0,75%	2	ON	/
	sacB-GFP (300ng)	0,75%	2,5	ON	/
	sacB-GFP (700ng)	0,75%	2,5	ON	/
	sacB-GFP (400ng)	0,75%	3	ON	/
	mCherry (400ng)	0,75%	3	ON	/
B. licheniformis	pUT-RFP3	/	2,5	3h	/
	pUT-GFP3	0,75%	2	ON	yes
	pUT-GFP3	/	2	ON	yes
	sacB-GFP (300ng)	0,75%	2,5	ON	yes
	sacB-GFP (150ng)	0,75%	2,5	ON	yes

Table 2. This table summarizes the results of genetic transformation in *Kocuria rhizophila*, *Bacillus subtilis*, and *Bacillus licheniformis* with various plasmids and experimental conditions (glycine concentration, voltage, recovery time), indicating successful transformation cases.

The results of the transformation experiments were remarkable (Table 2). *Kocuria rhizophila* demonstrated to be easier to be transformed with various plasmids, adapting the electroporation conditions according to the used plasmid. For example, with pUT-RFP3, this bacterium was efficiently transformed without glycine and with only three-hour of recovery time. Differently in the case of mCherry, we used 0.75% glycine concentration and extended the recovery overnight; in general, these experiments showed that longer recovery time and the use of glycine can aid in better transformation results.

Similarly encouraging results came from *Bacillus licheniformis*, considering that this strain was transformed with both the pUT-GFP3 and sacB-GFP plasmids. In this latter case, without glycine and at a concentration of 0.75%, the recovery was prolonged overnight. Such results suggest the experimental conditions set up were good enough to favour transformation in these strains and point out the importance of choosing the parameters appropriately.

Not all experiments produced positive results. The conditions tested were not able to transform *Bacillus subtilis*. Scientific literature refers to this strain for which, when a voltage of 7 kV is applied, one obtains effective results (Mohamadzadeh, 2024), a value that exceeds our electroporator, limited at 3 kV. This aspect underlines the need for adapting the experiment conditions depending on the requirements of bacterial strains and literature recommendations.

The aim of these genetic transformations is to study in detail the in vivo interactions between bacteria and plants. After obtaining the transformed bacteria, the strains were inoculated on the plants to visualise bacterial localization. The process of localization plays an important role in understanding the behaviour of bacteria in the ecosystem of plant roots. Using fluorescent markers, we were able to precisely monitor the presence of the bacteria in different areas: fluorescent bacteria were concentrated in the primary structure zone and close to the root tip (Figure 17 B), and they were primarily found in the rhizodermis, the root epidermis (Figure 17 A). This distribution highlights the potential role of the microbiome in promoting root development and protecting the plant suggesting that these microorganisms may actively interact with root cells in specific areas for nutrient uptake and growth.

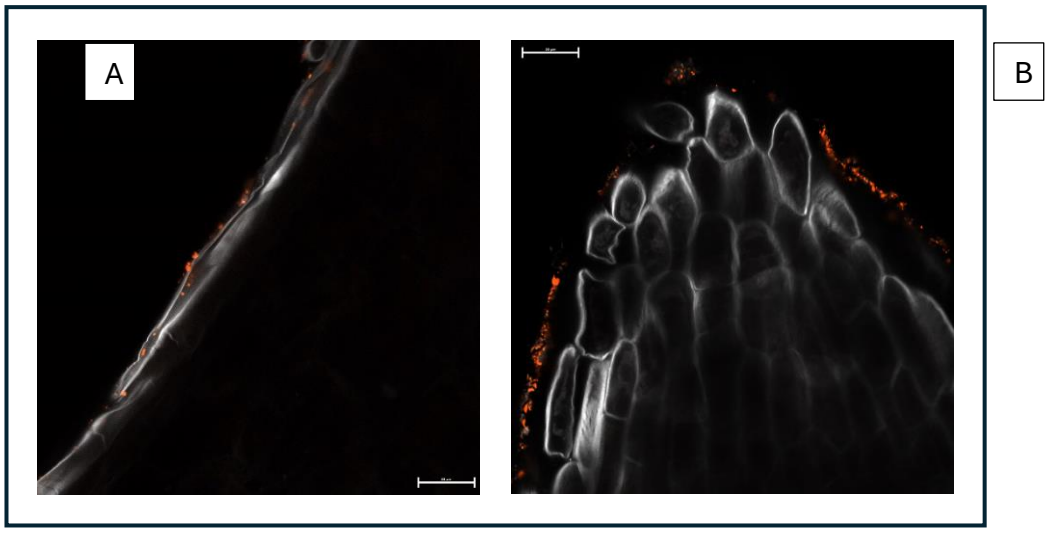

Figure 17. *K. rhizophila* transformed with RFP plasmid localised in the rhizodermis (**A**) and in the root tip (**B**), the scale bar is of 20 µm

The transformation efficiencies by different bacterial strains, as obtained from our experiment on bacterial transformation, have provided useful information and thus have laid a very good foundation for further studies that might be carried out on the interaction between bacteria and plants. This knowledge is particularly valuable for designing novel practices in sustainable agriculture and environmental biotechnology, offering new perspectives for application of bacteria in plant growth promotion and in plant protection against phytopathogens and abiotic stresses. Further research in this direction will be a very exciting and promising future frontier of both agricultural science and industry.

4. Conclusions

Rapidly increasing global temperatures and more frequent droughts have had a significant effect on food security and agriculture in recent years. In 2023, the global temperature rised to record levels relative to pre-industrial times, and drought-affected areas are spreading across all continents, putting crops in the Mediterranean at particular risk. For the future of agriculture and natural resources, one of the most urgent issues is the climate crisis, which is being made worse by population growth.
Along with climate change, intensive farming methods and excessive chemical use are deteriorating soil quality, which is causing microbial biodiversity—which is essential for plant health—to decline.

Beneficial microorganisms like plant growth-promoting rhizobacteria (PGPR) are important in this situation because they enhance nutrient uptake, encourage plant growth, and assist plants in surviving water stress. Compost, a resource that not only enriches the soil with nutrients but also microorganisms that can increase plant resilience to environmental stresses, is one of the suggested ways to counteract soil degradation and restore microbial biodiversity.

Given these problems and compost's potential, our study focused to understand how effectively composting soil can help plants overcome water stress, contributing to the development of more sustainable ways of managing natural resources and agriculture as a consequence of climate change.

Experiments conducted on drought-stressed plants and the study of bacterial growth curves provide several crucial insights into the interactions between compost microbes, plant health, and plant adaptability to environmental stresses. In particular, the results suggest that the compost microbiota could play a role in plant resilience, especially under moderate stress conditions. However, the specific contribution of different bacterial strains and soil conditions is not clear and needs further investigation, especially considering the possible loss of photosynthetic efficiency observed under certain experimental conditions.

In the first pilot, it was found that the control group also suffered a certain degree of stress due to the insufficient water supply, reducing the statistical validity of the data and confirming the importance of appropriate management of the experimental conditions. Subsequent experiments, in which irrigation levels were changed and compost, sterile compost and chemical fertilisers were added, confirmed that compost promotes better plant health, although without showing significant differences compared to sterile compost. This

raises questions about the specificity of the microbiota and the importance of the interaction between nutrients and microorganisms. In this regard, the role of compost bacteria in these dynamics should be further investigated, as some strains may not survive under adverse experimental conditions, such as compost sterilisation or the absence of sufficient resources to support their metabolic activity.

Another curious point that developed from the study concerns bacterial transformation and the role of compost bacteria in promoting plant wellbeing. The tested bacterial strains, counting *Kocuria rhizophila*, *Bacillus licheniformis* and *Bacillus subtilis*, appeared to have variable results in terms of transformation efficiency. For example, *Kocuria rhizophila* was particularly efficient in transformation without glycine, whereas using glycine improved the results for *Bacillus licheniformis*. However, *Bacillus subtilis* showed considerable difficulty in transformation, which can be attributed to the fact that the voltage applied during electroporation (maximum 3 kV) was insufficient to achieve efficient transformation, as indicated in the literature, where 7 kV is recommended. This point underlines the significance of adjusting experimental protocols to the specific characteristics of bacterial strains.

The ability to monitor the interaction between transformed bacteria and plants through fluorescent markers has opened up new viewpoints for studying bacterial behaviour in the rhizosphere and inside plant roots. This may give a more precise understanding of bacterial colonisation dynamics and their impact on plant well-being and development. In any case, the question remains that a few compost bacteria may not survive during the tests, especially under high-stress conditions or without an appropriate substrate, such as sterile compost. This factor may negatively influence the overall results by decreasing the number of live bacteria and hence their ability to provide benefits to plants.

In conclusion, the results give important insights for future investigate on the interaction between soil organisms and plants, with suggestions for the development of more sustainable agricultural practices. Differences in plant response to different treatments show the importance of an active and diverse microbiota and underline the complexity of these interactions. Further studies could focus on improving experimental techniques and identifying critical factors influencing the survival and activity of compost bacteria, especially under conditions of water or nutrient stress, to optimise biotechnological and environmental applications for the promotion of plant health and sustainable management of water resources in agriculture.

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