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Salomé Lengrand

Influence of seed-transmitted endophytic bacteria and humic substances in tomato osmotic stress response

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PhD Dissertation Committee

Co-supervisors:	Anne Legrève, UCLouvain
	Xavier Draye, UCLouvain
Jury Members:	Stanley Lutts, UCLouvain
	François Renoz, UCLouvain
	Patrick du Jardin, Gembloux Agro-Bio Tech, ULiège
	Stéphane Compant, Austrian Institute of Technology
President:	Stéphane Declerck, UCLouvain

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Abbreviations

ABC: ATP-binding cassette
ABA: abscisic acid
ACC: 1-aminocyclopropane-1-carboxylate
ALK: alkaloids
ANAMMOX: anaerobic ammonium oxidation
ANOVA: analysis of variance
ANOSIM: analysis of similarities
AOB: ammonium-oxidizing bacteria
APX: ascorbate peroxidase
ASV: amplicon sequence variants
BH: Benjamini-Hochberg
BLAST: basic local alignment search tool
BCYE: buffered charcoal yeast extract
CAT: catalase
CAS: chrome azurol s
CDPK: calcium-dependent protein phosphorylation
CEC: cation exchange capacity
CK: cytokinins
CLSM: confocal laser scanning microscopy
CTAB: cetyltrimethylammonium bromide
DAP: diaminopimelate
eDNA: environmental DNA
ET: ethylene
EPS: exopolysaccharides
FA: fulvic acids
FISH: fluorescence *in situ* hybridization
FV/FM: maximum quantum yield of photosystem II
GA: gibberellic acids
GB: gibberellin
GPOX: glutathion peroxidase
HA: humic acids
HCN: hydrogen cyanide
HS: humic substances
HTS: high-throughput sequencing
IAA: indole-3-acetic acid
JA: jasmonic acid

LBA: luria broth agar
LPR: root hydraulic conductivity
MDA: malondialdehyde
MFS: major facilitator superfamily
MLSA: multi-locus sequence analysis
MS: Murashige and Skoog
MFP: membrane fusion protein
NA: nutrient agar
NCBI: National Center for Biotechnology Information
NO: nitric oxide
NR: nitrate reductase
OTU: operational taxonomic unit
PA: polyamines
PAL: phenylalanine ammonia-lyase
PEG: polyethylene glycol
PCOA: principal coordinates analysis
PNA: peptide nucleic acid
POX: peroxydase
PM: plasma membrane
PBS: phosphate-buffered saline
PERMANOVA: permutational multivariate analysis of variance
PGP: plant growth promoting
POD: peroxidase
PVP: polyvinylpyrrolidone
QPCR: quantitative polymerase chain reaction
QPSII: actual quantum yield
RAPD: random amplified polymorphic DNA analysis
RND: resistance nodulation and cell division
ROS: reactive oxygen compounds
RRN: ribosomal RNA
RWC: relative water content
SA: salicylic acid
SDG: sustainable development goals
SEM: scanning electron microscopy
SOD: superoxide dismutase
SOM: soil organic matter
T6SS: type VI secretion system
TAL: tyrosine ammonia-lyase

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Glossary

Certain terms commonly used in the biosciences can vary in meaning depending on the specific field in which they are applied. Therefore, it is helpful to define some key concepts as they are used throughout this thesis.

- **Complementarity** occurs when two or more elements (such as substances, processes, or actions) work together in a way that enhances the overall outcome, but each element contributes its own distinct function or benefit without directly enhancing each other's effects. The outcome is additive.

- **Synergism** is a coordinated or correlated action of two or more structures, agents, or physiologic processes so that the combined action is greater than the sum of each acting separately. The outcome is multiplicative (Thoo et al., 2013; Sonam and Guleria, 2017; Tavadyan and Minasyan, 2019; Olszowy-Tomczyk, 2020).

- The **endophytome** is the collective community of endophytes, including bacteria, fungi, archaea, viruses, and other microbes, such as protists, protozoa, or archaebacteria, that inhabit plants as obligatory or facultative organisms that are vertically or horizontally transmitted (Lengrand et al., 2024).

- **Mutualism** is a type of symbiotic relationship between two different species where both parties benefit from the interaction. Each organism gains advantages that enhance its survival, growth, or reproductive success due to the presence of the other (Holland and Bronstein, 2008).

- **Resilience** is a performance measure representing the system ability to survive disruptive events, and the rapidity in restoring system capacity after the disruptive event has occurred (Caputo et al., 2019).

- **Symbiosis** is a broader term that describes any close, long-term interaction between two different biological species. This relationship can be beneficial, neutral, or harmful to one of the partners

(Martin and Schwab, 2012a). The name symbiosis was coined by Anton de Bary (1879) and he defined it literally as the "living together of dissimilarly named organisms." (Martin and Schwab, 2012b).

GENERAL INTRODUCTION

1. Problem statement

Climate change is intensifying extreme weather events such as heat waves, droughts, and shifts in rainfall patterns, posing significant threats to global food security. These events can drastically reduce crop yields, which is particularly concerning given the growing global population projected to reach nearly 10 billion people by 2050 (Singh et al., 2023; Searchinger et al., 2019). To meet this demand, food production must increase substantially, necessitating innovative and sustainable agricultural practices (Espinosa-Leal et al., 2018; Raza et al., 2019; Qaim, 2020). Among the main crops, tomato, *Solanum lycopersicum* is particularly vulnerable, with risks of yield reduction of up to 40% due to more frequent droughts occurrences (Hammond et al., 2022; Kopecká et al., 2023).

The European Union has taken proactive steps to address these challenges through initiatives like the Green Deal and the Farm to Fork Strategy. These policies aim to reduce the use of chemical pesticides and fertilizers, promote sustainable agricultural practices, and align with the Sustainable Development Goals (SDGs) (Commission, 2020). These initiatives highlight the need for innovative solutions that can enhance crop resilience and productivity while minimizing environmental impact.

Biostimulants, substances designed to enhance plant nutrition, stress tolerance, and quality traits, have gained increasing interest as potential solutions (du Jardin, 2015). Among these substances, humic substances (HS), which are major components of soil organic matter from decomposed plant, animal, and microbial residues, are particularly noted for their role in enhancing plant resilience against abiotic stresses (du Jardin, 2015; Rouphael and Colla, 2018). They can directly improve

plant growth by activating essential hormonal and antioxidant pathways in plants (Piccolo, 2002; Canellas et al., 2015).

Our understanding of how HS influence plant growth and stress response is still incomplete, particularly when viewing plants as holobionts—a concept that recognizes plants not just as individual entities but as complex systems comprising both the plant and its microbiota (Hassani et al., 2018; Lemanceau et al., 2017). This holistic vision is essential for understanding how plants, in interaction with endophytes, manage stresses (Bary et al., 1866; Reinhold-Hurek and Hurek, 2011). Some endophytes play pivotal roles in enhancing plant health, including improving nutrient absorption and increasing resistance to environmental stressors (Hardoim et al., 2015; Pandey et al., 2019; Eid et al., 2021; Santoyo et al., 2016). These microbial partners are either vertically transmitted through seeds, ensuring continuity from one plant generation to the next, or horizontally acquired from the surrounding environment, adding diversity to the plant's internal ecosystem (Truyens et al., 2015; Shade et al., 2017; Frank et al., 2017; Rochefort et al., 2021).

Endophytic bacteria, particularly seed-transmitted endophytes, are crucial in the early stages of plant development and overall health (Shahzad et al., 2018; Pandey et al., 2022). Despite their significance, our knowledge of seed-transmitted endophytic bacteria remains sparse (Simonin et al., 2022).

Although the crucial role of these microbial communities in plant physiology, studies investigating the effects of HS on these communities under varying stress conditions remain less explored. This gap underscores the necessity for more comprehensive studies that utilize multiple approaches, such as metagenomics, culturomics, metaproteomics and metatranscriptomics. These methods can help elucidate the complex relationships within plant holobionts, potentially leading to more effective biostimulation strategies.

The aim of this thesis is to deepen our knowledge of tomato endophyte communities and to evaluate their role under stress conditions.

2. Thesis outline

In this thesis, we begin with a literature review (Chapters I to IV). In Chapter I, we first introduce the concept of endophytes and endophytomes, exploring their composition and formation, including the modes of transmission and common genomic characteristics of endophytes. Additionally, we present the impact of biotic and abiotic factors on endophytomes. Next, in Chapter II, we explore the role of endophytic bacte-

ria in increasing plant drought stress tolerance. In Chapter III, we focus on seed-transmitted endophytic bacteria, discussing their relevance and the composition of the seed bacterial endophytome. We also examine the evolution of bacteria present in seeds across different plant structures and specifically investigate the seed-borne bacterial endophytes of *S. lycopersicum*.

Chapter IV explores the mechanisms of action of HS used as biostimulants, integrating both the indirect and direct effects of these substances on plant metabolism, including their interactions with hormonal and reactive oxygen species (ROS) pathways. The chapter also examines the complementation effects of bacteria and HS and their interactions with plant-associated microbiota in hydroponic systems.

In the first part of the results, presented in Chapter V, we provide insights into seed-borne endophytic bacteria of *S. lycopersicum* var. Moneymaker. We detail the dynamic colonization processes and their implications for tomato tolerance to osmotic stress. Chapter VI focuses on how HS increase tomato tolerance to osmotic stress while modulating vertically-transmitted endophytic bacterial communities. We discuss the impact of HS on plant growth under both non-stress and osmotic stress conditions and analyze the shifts in endophytic bacterial community composition induced by these factors.

Chapter VII is devoted to a general discussion, where we summarize the main results and highlight the methodological advances made in our approach. We discuss the complex interplay between plant genomes, environmental factors, and microbiota in defining plant phenotypes. Additionally, we address the challenges of identifying endophytic communities and propose future research directions. We conclude by discussing the potential applications of biostimulants and endophytic bacteria in agricultural and political contexts, emphasizing their significance for sustainable agriculture.

LITERATURE REVIEW

CHAPTER I. Plant-endophytes interactions

The first chapter of this thesis is devoted to introducing the reader to the concept of endophytes and the dynamic interaction that occurs between plants and their endophytome, modulating their development and resistance to biotic and abiotic factors. This chapter revisits and refines the definitions of endophyte and endophytome, focusing on the complex relationships within bacterial endophytomes. It also explores the composition and formation of the endophytome, focusing on the mode of transmission and the genes commonly found in genomes of endophytes. Finally, the impact of biotic and abiotic factors on endophytomes is presented, highlighting the adaptability and resilience of plant-associated microbes.

Bacterial endophytome sources, profile and dynamics – a conceptual framework

Salomé Lengrand[1†], Lena Pesenti[1†], Claude Bragard[1], Anne Legrève[1*]

[1] Université catholique de Louvain (UCLouvain), Earth and Life Institute Applied Microbiology (ELIM), Croix du Sud 2 bte L7.05.03, 1348 Louvain-la-Neuve, Belgium

[†]These authors contributed equally to this work and share first authorship.

[*]Correspondence: Anne Legrève (anne.legreve@uclouvain)

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1. Abstract

Currently, it seems inconceivable to dispute the major role of microorganisms in human health or insects with endosymbionts. Although microbial endophytes were discovered long ago, little is known about the roles of plant-associated microorganisms. Some endophytes are horizontally transmitted, whereas others are seed-borne; together, they influence plant health. Beneficial endophytes can promote plant growth and yield by increasing plant resistance to biotic and abiotic stresses. Recently, the tools available to study the phytobiome have much improved, opening doors for a better understanding of the fascinating interactions taking place at the plant level. This review redefines the conceptual framework for "endophyte" and "endophytome," focusing on the intricate dynamics of bacterial endophytomes. Systematically examining the formation pathways and profiling endophytes allows for a comprehensive exploration of the intricate dynamics governing plant-microbe interactions. Additionally, the assessment of how endophytomes are influenced by both biotic and abiotic factors provides essential insights

into the adaptability and resilience of plant-associated microorganisms. Our comprehensive analysis integrates genomic insights with environmental considerations, offering a nuanced perspective on the functional roles of bacterial endophytomes. Therefore, a new, inclusive definition is essential to accurately represent the complexity of interactions within the plant microbiome as well as having the whole picture of associated concepts.

2. Introduction

Plants naturally host distinct microbial communities outside and inside their tissues in the phyllosphere (i.e., air-plant interface), rhizosphere (i.e., root-soil interface), and endosphere (i.e., internal tissues of the plant). Microorganisms associated with the endosphere, known as endophytes, directly influence plant metabolism and plant cells. Several studies have highlighted the benefits of endophytes in improving plant health (Harman et al., 2021) by revealing their ability to enhance nutrient uptake, disease resistance, stress tolerance, conserve water, and promote soil health, which contributes to reducing environmental impacts, increasing productivity, and long-term sustainability in agriculture (Compant et al., 2010b; Hardoim et al., 2008, 2015). Therefore, harnessing these natural benefits as biocontrol agents can reduce environmental impacts of pest management, offer targeted pests and disease control, and enhance agricultural system resilience.

In endophyte studies, conventional methods often entail isolating bacteria from culture media after surface disinfection, followed by reinoculation into plants. A critical issue with this approach is its heavy reliance on cultivating endophytes in artificial culture media, despite the majority of the microbial diversity in natural environments remaining unculturable. Additionally, the surface disinfection step used to isolate endophytes introduces biases limiting the recovered isolates' diversity. Therefore, the isolated strains might not accurately represent the full diversity of plant-associated endophytes. Furthermore, reinoculation of endophytes from one plant into another of the same species, as practiced in the conventional approach, disrupts natural host-specific interactions between endophytes and their host plants. These knowledge gaps hamper our ability to understand the ecological significance and functional potential of endophytes fully (Compant et al., 2019; Hardoim et al., 2015; Papik et al., 2020).

Given these limitations, there is an urgent need for a timely review and re-evaluation of the conventional methods employed in endophyte research. Emerging techniques such as metagenomics, metatranscriptomics, and single-cell sequencing can provide valuable insights into the

diversity, functionality, and dynamics of endophytes without the constraints of cultivation-based methods. A more effective approach would be directly examining the endophytic communities to evaluate how abiotic and biotic stressors influence their dynamics and evolution. Rapid progress in sequencing techniques has enabled the *in situ* study of these endophytic communities, thereby advancing the understanding of their functional roles in plants. Although progress has been made on plant endophytes, there is still a long way to go to achieve what has been done so far on human microbiota (Gilbert et al., 2018)

Here, we discuss the multifaceted nature of plant-endophyte interactions, redefine the concept of endophytes, explore the formation of the bacterial endophytome, examine the existence of a bacterial endophytome profile, analyze the factors driving variation in endophyte communities. This will provide a conclusion and future perspectives on the topic and highlight how understanding the interactions between plants and endophytes can contribute to sustainable agriculture.

3. Plant-endophyte multifaceted relations

3.1. Back to the roots – defining endophyte

The term 'endophyte' is derived from the Greek words 'endon' meaning 'within' and 'phyton' meaning 'plant'. The term was coined by Bary et al. (1866) referring to the presence of 'bacteria' living inside a fern called *Ophioglossum*. At that time, the term 'bacteria' was used to encompass all living organisms, including those living inside a plant.

Endophytes have been defined thereafter in different ways, not always including the full range of living organisms able to reside within their host plants. Petrini (1991) proposed restricting the definition to 'all organisms inhabiting plant organs that, at some time in their life, can colonize internal plant tissues without causing apparent harm to the host'. Several other definitions have been proposed, often restricting endophytes to a group of organisms (e.g., fungi or bacteria), symbiotic and/or commensal microorganisms, or facultative and/or mandatory microorganisms (Cabral et al., 1993; de Medeiros Azevedo et al., 2021; Hardoim et al., 2015; Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2006).

Recent technological advances, facilitated by metagenomic studies and drastic reduction in sequencing costs, have provided a much better view of the diversity and the type of microorganisms associated with plants, with emerging terms like 'phytobiome' referring to the collec-

tive community of microorganisms, including bacteria, fungi, archaea, viruses, and other microbes, that interact with plants within a specific environment. It encompasses a plant-associated microbiome that includes both endophytic and rhizospheric microorganisms. The term 'holobiome' is defined as the collective assemblage of all organisms, including plants, animals, and microorganisms, as well as their genetic material, that coexist within a specific ecological niche or habitat. Redefining the term 'endophytome' with regard to these newly defined terms is also necessary for the precision, clarity, and elucidation of the complex interactions and synergistic relationships between organisms, as well as their combined contributions to ecosystem functioning, health, and resilience.

Several plant-associated microbes may sometimes cause disease but stay associated with their host plants simply as commensals. For example, *Xylella fastidiosa* has been associated with more than 600 asymptomatic host plants (EFSA Panel on Plant Health et al., 2019). Similarly, *Clavibacter* and *Curtobacterium* are often associated with asymptomatic plants, although some species are dangerous plant pathogens (Eichenlaub and Gartemann, 2011; Bulgari et al., 2014). Using a metagenomic approach to search for plant viruses has revealed the presence of numerous viral sequences and viruses that do not cause diseases (Roossinck, 2010). Some plant pathogens, individuals of the same species, or even strains, are also found in alternate hosts, where they do not cause diseases (Salvaudon et al., 2005). What drives the shift of such organisms along the 'parasite–mutualist continuum' has yet to be explained (Drew et al., 2021). Dependent on the host-microorganism association, excluding the 'pathogen' from the endophyte definition is impossible considering these examples.

To fully capture the complexity and diversity of endophytic organisms and their relationships with plants and elucidate the complex network of interactions and dependencies in endophytic microbial populations in plants, there is an urgent need to return to the original definition proposed by Bary et al. (1866). As our understanding of endophytes continues to evolve, it is essential to develop a comprehensive and inclusive definition encompassing all aspects of their ecology and biology.

Based on the definition of the human microbiome, we propose to define the "endophytome" as a collective community of endophytes, including bacteria, fungi, archaea, viruses, and other microbes, such as protists, protozoa, or archaebacteria, that inhabit plants as obligatory or facultative organisms that are vertically or horizontally transmitted. The endophytome is composed of endophytes that interact dynamically with plants according to six types of interaction (Kogel et al., 2006; Papik et al., 2020; Swanson et al., 2022; Agler et al., 2016; Berendsen et al., 2018) (Figure I-1). A given endophyte may develop different interactions

with its host during its life cycle.

Mutualism - mutually beneficial relationships or interactions between endophytes and their host plants, in which both partners derive advantages and receive reciprocal benefits. Example: Arbuscular mycorrhizae fungi (from *Glomeromycota* phylum) form structures called arbuscules inside the plant root cells, facilitating carbohydrate exchange and enhancing the plant's ability to absorb water and nutrients (Sturmer and Kemmelmeier, 2021).

Parasitism/ "rhizophagy" - where only one actor benefits that can lead to plant disease, while plants can benefit microorganisms without providing any advantages. Example : rhizophagy is a mechanism where plant can internalize and digest nutrients from symbiotic microbes (bacteria and fungi) (White et al., 2018).

Commensalism - where it is beneficial for one agent and safe for the other. Example : Fungal endophyte *Epichloë* residing within the tissues of grass can produce secondary metabolites, such as alkaloids beneficial for plant host (Bastias et al., 2017).

Neutralism - where the interaction does not influence either partners.

Competition - where both partners are negatively affected by the interaction. Example : *Xylella fastidiosa* is a bacterium that colonises the xylem vessels, the water-carrying channels in the plant. It depends on the plant for its nutrition and survival. When it rapidly kills the host plant, the source of nutrients on which the bacterium depends is rapidly exhausted. This can lead to a significant halt or slowdown in bacterial growth (Landa et al., 2022).

Ammensalism - where one organism is negatively affected or inhibited, whereas the other remains unaffected.

A "healthy" endophytome refers to the absence of any disease and shows a large degree of diversity even depending on the host (genera, species), the stage of development, and the environment. The endophytome can also be characterized as a functional core or a complement of metabolic and other molecular functions performed by the microbiome with a plant part but not necessarily provided by the same organisms in different species or individuals (Lloyd-Price et al., 2016).

The following sections focus on bacterial endophytomes. Our focus will now shift toward unraveling the entry pathways of bacterial endophytes and exploring whether transmission occurs through vertical or horizontal pathways. We will explore the drivers of the diversity and composition of bacterial endophytes. By unraveling the mystery of the bacterial endophytome, we aimed to gain deeper insights into its

functional roles and potential applications in agriculture and ecosystem management.

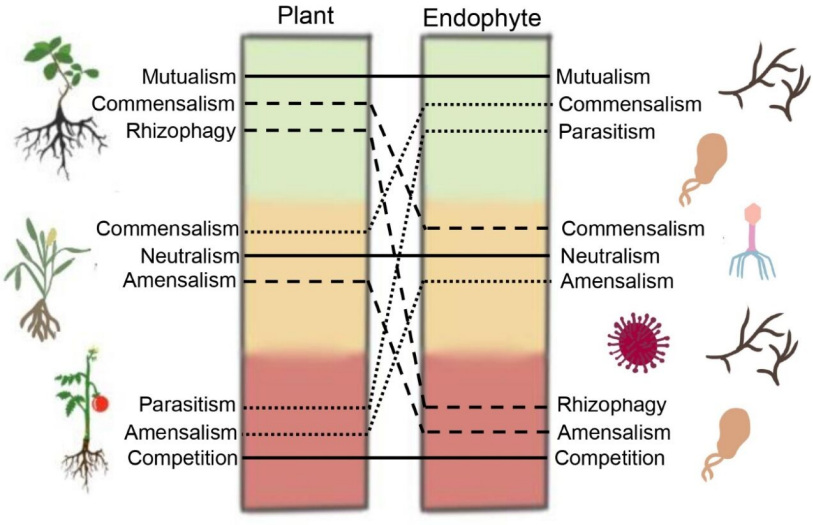

Figure I-1: Theoretical framing of interactions between endophytes and host plants.

Some interactions are more evident than others and therefore more studied (e.g. mutualism). Others are more complex to represent or even name (e.g. neutralism). Mutualism – mutually beneficial interaction between endophyte and its host plant. Both partners derive advantages and receive reciprocal benefits. Parasitism / "rhizophagy" - only one actor benefits. This can lead to plant disease or plants can benefit microorganisms without providing any advantages. Commensalism - beneficial for one agent and safe for another. Neutralism – no influence on either of the partners. Competition - negatively affects both partners. Ammensalism - one organism is negatively affected or inhibited, while the other organism remains unaffected. A given endophyte may develop different interactions with its host during its life cycle. The two central rectangles show the effect of the interaction on the plant (left) and the endophyte (right). Green, yellow or red represent beneficial, neutral or harmful effects of the interaction. Each line represents a type of interaction.

3.2. Where does bacterial endophytome come from ?

The endophytome comprises seed-transmitted microorganisms and microorganisms from the environment. Endophytes colonize the host vertically from parent to offspring, mainly via seed or vegetative propagation, horizontally through the environment, or in mixed modes (Bright and Bulgheresi, 2010) (Figure I-2).

Vertical pathway: This pathway involves the transmission of bacteria through seeds or vegetative propagation (Cankar et al., 2005; Mastretta

et al., 2009; dos Santos et al., 2018). Endophytes present in the seeds can be acquired from pollen, flowers, fruits, or the plant's soil. Bacteria reach the reproductive organs from the seed via xylem vessels or the shoot apical meristem and differentiate into reproductive organs (Frank et al., 2017). Most vertically transferred bacteria must spend their entire lives inside the host, on which they depend to survive. Vertical transmission has been identified as a factor that helps align the interests of mutualists, suggesting that symbiotic endophytes transferred vertically are mutualists and likely provide an indispensable function (Herre et al., 1999). Indeed, seed endophytes have been shown to benefit their hosts by releasing seeds from dormancy, facilitating germination, protecting against pathogens, and promoting the growth of seedlings (Puente et al., 2009). Typical members include the genera *Acinetobacter*, *Bacillus*, *Micrococcus*, *Paenibacillus*, *Pantoea*, and *Pseudomonas* (Truyens et al., 2015).

Horizontal pathway: Horizontal transfer is mainly realized by the soil-to-root route because roots are considered the main entry point for microorganisms. Indeed, bacteria can penetrate the endodermis, the innermost layer of the root cortex, through root hair channels, cracks in the epidermis, cell-to-cell junctions, or through root cap cells that detach as the roots grow (Hardoim et al., 2008). Once bacteria reach the endodermis, they must pass through the Casparian strip, a cell wall thickening that prevents the free diffusion of molecules and ions between the root cortex and vascular tissues. Studies suggest that some bacteria can pass through the Casparian strip by inducing the expression of endodermal transporters, such as aquaporins, which allow bacteria to cross the endodermal barrier (Bulgarelli et al., 2013). Additionally, some bacteria secrete cell wall-degrading enzymes or manipulate host cell signaling pathways to facilitate their entry into the endodermis. Once in the endodermis, endophytes mainly move into the xylem vascular system, allowing systemic colonization of internal plant compartments (James et al., 2002). They can colonize xylem vessels and perforation plates between these vessels (James et al., 2002; Compant et al., 2005). In addition to this main transport route, other endophytes also colonize intercellular spaces (Hardoim et al., 2015). This pathway can be considered passive or active (Hardoim et al., 2008) depending on the penetration mode within the plant. An active penetration pathway can be mediated by the attachment of bacterial endophytes to plant cells using their flagellum and the secretion of metabolites that facilitate the penetration process (mostly exopolysaccharides [EPS] and cell wall degrading enzyme (Kandel et al., 2017; Pinski et al., 2019; Ullah et al., 2019a). To some extent, root exudates and rhizodeposits can attract certain types of microorganisms *in planta* (Cocking, 2003; Compant et al., 2010a; dos Santos et al., 2018; Germida et al., 1998; Hallmann et al., 1997; Hardoim et al., 2015; Sessitsch et al., 2002). Bacterial endophytes can also be horizontally transmitted to the phyllosphere

level (Redford et al., 2010; Kembel et al., 2014; dos Santos et al., 2018) from dust, rainwater, or surrounding pollinators. Hydathodes, lenticels, and stomata are the primary gateways for endophytes in these environments (Compant et al., 2010a; Frank et al., 2017; Hardoim et al., 2015). Plants can close or open their stomata upon contact with different microbial antigens (Gimenez-Ibanez et al., 2017; Melotto et al., 2017), which suggests that the plant immune system plays a role in the selection of microbes entering the endosphere (Fesel and Zuccaro, 2016). However, some foliar endophytes also control stomatal opening (Rho et al., 2018). Plant- and sap-feeding insects can be endophyte vectors. These insects have piercing–sucking mouthparts and can directly puncture the phloem and/or xylem cells that transmit the endophytes (Frank et al., 2017). Finally, only compatible endophyte-plant interactions can successfully colonize the endosphere, which explains the reduced number of endophytes compared with rhizospheric bacteria (Hardoim et al., 2015; Khare et al., 2018). An analysis of all sequenced 16S genes available in the International Nucleotide Sequence Database Collaboration identified four phyla containing more than 96% sequenced endophytic prokaryotes: Proteobacteria (54%), Actinobacteria (20%), Firmicutes (16%), and Bacteroidetes (6%). Most endophytic bacteria belong to the class *Gammaproteobacteria*, including *Acinetobacter*, *Enterobacter*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, and *Serratia* (Hardoim et al., 2015).

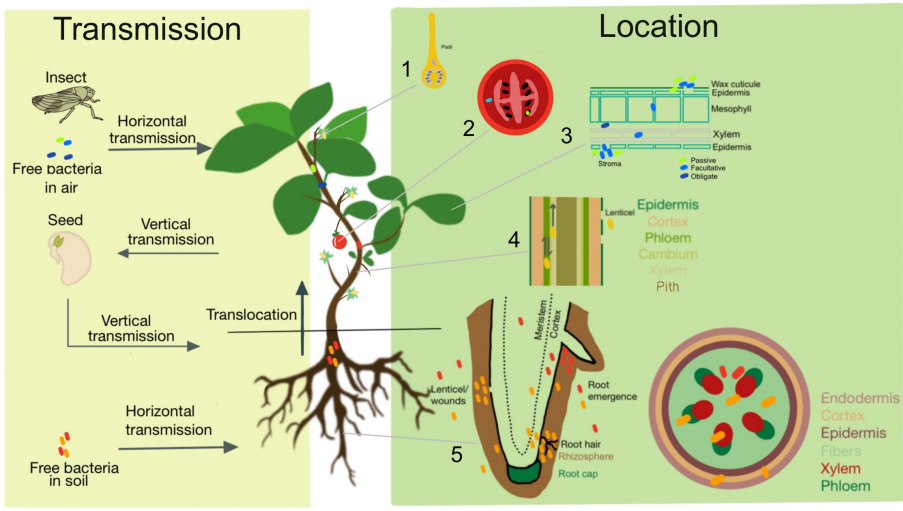

Figure I-2: Types of endophyte transmission and endophyte location in plants.

The gray arrows indicate different types of transmission of endophytes in the host plant: horizontally and vertically. The endophytes can move through translocation to reach different locations within the plant: inside the flower (1), of the fruit (2), of the leaves (3), of the shoot (4), and of the roots (5).

3.3. Toward an endophytome profile?

The endophytome comprises various endophytes characterized by genomic differences compared with free-living microorganisms. However, these differences can be difficult to describe because some are facultative (del Carmen Orozco-Mosqueda and Santoyo, 2021). Ali et al. (2014) were the first to compare the genomes of endophytes and non-endophytes, thereby identifying the genes involved in secretion and transport activities, polymer degradation or modification, detoxification, and maintenance of redox potential. They suggested that these functions might play a role in endophytic behavior, allowing them to colonize plants.

This review presents the functional characteristics of endophytic bacteria and their involvement in plant colonization and propagation. The soil-to-root colonization steps were divided into: (1) detecting root exudates, (2) motility toward the plant, (3) adhering to root surfaces, (4) penetrating the root surface, and (5) colonizing the plant's internal parts. Each of these stages is mediated by a variety of biomolecules that influence the expression of both bacterial and colonized plant genes (Pinski et al., 2019) (Figure I-3).

Colonization by endophytic bacteria starts with the chemotaxis of free-living bacteria toward the roots, aided by methyl-accepting chemotaxis proteins (MCPs). These transmembrane sensors detect molecules and direct bacteria toward attractants or away from repellents (Balsanelli et al., 2016). The involvement of MCPs in plant colonization was demonstrated using inactivation mutants of *Herbaspirillum seropedicae* SmR1 and *Azospirillum brasilense* Sp7. In SmR1, one MCP is required to sense the rhizosphere and direct bacteria toward host-secreted compounds. In Sp7, the inactivation of another MCP results in impaired chemotaxis and colonization of plant roots (Greer-Phillips et al., 2004).

At the root surface, bacteria can attach through biofilms, which contain water, proteins, polysaccharides, eDNA, RNA, and ions. Mutants in various species, such as *G. diazotrophicus* PAL, have shown that other polymers, including EPS, glucomannan, and cellulose, also affect attachment and colonization. Adhesion is mediated by the flagella, pili, curli, and hemagglutinins. The upregulation of genes encoding filamentous hemagglutinin proteins suggests their involvement in root attachment (Barak et al., 2005; Williams et al., 2008; Monteiro et al., 2012; Ainelo et al., 2018). Bacterial surface components, particularly lipopolysaccharides (LPS), play crucial roles in early attachment and colonization. Mutations in *rfbB* or *rfbC*, which are involved in rhamnose biosynthesis, reduce the attachment to and colonization of maize roots by *H. seropedicae* SmR1, as well as the bacterium's robustness against detergents, antibiotics, and phytohormones. Similarly, mutations in the *rfbD* gene of

A. brasilense impair attachment and colonization efficiency. O-antigen ligase, involved in O-antigen biosynthesis, was upregulated during colonization, indicating its involvement in plant-endophyte interactions. Bacterial LPS binds to maize root lectin proteins leading to agglutination, which is impaired when the O-antigen ligase is inactive, resulting in reduced attachment and colonization efficiency (Jofre et al., 2004; Balsanelli et al., 2013).

After attachment to the root surface, bacterial endophytes enter the plant through natural openings or wounds or by producing plant cell wall-degrading enzymes (e.g., pectinase or cellulase) that facilitate entry and spread within plants. For example, *Azoarcus* sp. BH72 requires endoglucanase for rice root colonization and spreads to the aboveground parts, whereas *Bacillus mycoides* EC18 upregulates hydrolases and chitin-binding proteins in response to root exudates (Badri and Vivanco, 2009; Reinhold-Hurek and Hurek, 2011; Baetz and Martinoia, 2014).

During colonization, specific endophytic genes, such as genes encoding membrane associated proteins belonging to the resistance nodulation and cell division (RND) efflux systems, actively contribute to successful establishment. Among them, the Membrane Fusion Protein (MFP) subunit of the RND family efflux systems has been shown to have a significant role in the successful colonization of the host plant, as observed in the case of the endophyte *Erwinia amylovora* in apple trees (Taghavi et al., 2010). Detoxification mechanisms such as glutathione synthesis and reductase-related genes have been identified as crucial factors for competent endophytes, as they protect their host against oxidative stress induced after host infection (Compant et al., 2010a). Thus, these genes play a potential role in the protective response of endophytic bacteria to oxidative stress resulting from the infection of plant hosts (Alqueres et al., 2013).

After entering the plant, certain endophytes spread systemically throughout the plant, eventually reaching the flowers, fruits, and seeds through the xylem vessels of their host plants, taking advantage of their flagella and the plant's transpiration stream (Compant et al., 2005).

Transport proteins, including various genes associated with the major facilitator superfamily (MFS) transporter system, play a crucial role in the endophytic lifestyle by facilitating the uptake of plant-synthesized nutrients. These proteins are essential for acquiring nutrients found internally within plants or released into the rhizosphere. Their primary function involves the exchange of diverse carbohydrates and amino acids, enabling efficient nutrient transfer between the endophyte and its host plant (Taghavi et al., 2010; Ali et al., 2014). Certain endophytic genes appear responsible for bacterial nutrition within the plant, including

non-plant-cell-wall-breaking hydrolases found in the genomes of plant growth-promoting bacterial endophytes, such as *Enterobacter* spp. 638 and *Serratia proteamaculans* 568 (Taghavi et al., 2010). The widespread presence of these enzymes among different endophytes suggests their potential involvement in the versatile utilization of sugars, which could be a valuable characteristic of proficient endophytes.

Endophytic bacteria possess various secretion systems that differentiate them from free-living bacteria. Among these, the type VI secretion system (T6SS) appears to be involved in utilizing plant carbon sources (del Carmen Orozco-Mosqueda and Santoyo, 2021). Bacterial endophytes commonly exhibit type I and II secretion systems (Reinhold-Hurek and Hurek, 2011), whereas type III and type IV secretion systems are predominantly found in pathogenic bacteria and are typically absent in endophytes (Downie, 2010). The type V secretion system, also known as an autotransporter, is primarily observed in endophytes. Additionally, T6SS may benefit plant-microbe interactions (Mattinen et al., 2008) and have been detected in certain bacterial endophytes (Reinhold-Hurek and Hurek, 2011).

Some groups of genes have been identified as conserved and potentially responsible for endophytic behavior, including various transcriptional regulators involved in metabolic adaptation and quorum sensing, such as *AraC*, *FrmR*, *AsnC*, *LrgB*, *LysR*, *DeoR*, and *WrbA* (Table I-1). Many of these regulators play a role in modulating carbohydrate metabolism, which is crucial when cells enter the stationary phase of growth. The presence of these regulatory proteins suggests their likely function, specifically in the non-invasive breakdown of the plant cell wall, which occurs when endophytes meet plant hosts during infection. Additionally, some of these transcriptional regulators are involved in communication pathways that are vital for altering the behavior of an organism when it adapts to its physiology (Ali et al., 2014). The gene encoding a lysine biosynthesis enzyme, specifically diaminopimelate decarboxylase, along with the gene encoding a lysine exporter protein, may also be involved in the transition of the bacterium from a free-living state in the soil to an endophytic state within the plant (Maddocks and Oyston, 2008; Ali et al., 2014).

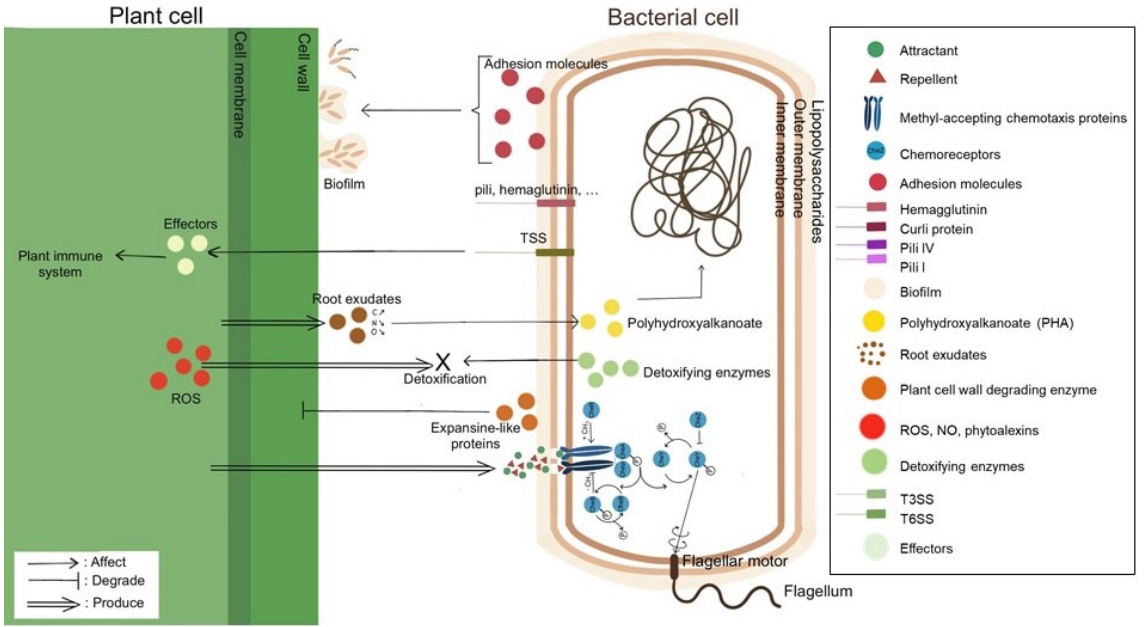

Figure I-3: Principal drivers involved in bacterial endophyte colonization of plant host.

Adhesion molecules and hemagglutinin, curli protein, pili IV, and pili I facilitate the attachment of endophytic microorganisms to the plant's surfaces, promoting biofilm formation and providing a stable environment for colonization, nutrient exchange, and interactions within the plant tissues. Type 3 and 6 secretion systems produce effectors, which play a role in manipulating the host plant's cellular processes, suppressing immune responses, and promoting colonization. Plant cell wall degrading enzymes enable endophytes to break down and penetrate the plant cell wall, facilitating colonization. Expansin-like proteins promote cell wall loosening and expansion, allowing endophytes to access nutrients and establish symbiotic interactions within plant tissues. Detoxifying enzymes can neutralize reactive oxygen species generated by plants as a defense response and metabolize phytoalexins, aiding in the plant's protection against oxidative damage and enhancing its resistance against pathogens or stressors

Table I-1: Conserved classes of genes responsible for endophytic behavior, including various transcriptional regulators.

Abbreviation	Function	Reference
AraC	Involved in carbon metabolism, stress responses, and virulence management	(Gallegos et al., 1997; Martin and Rosner, 2001)
FrmR	Involved in the global transcriptional regulator and negatively controls cellular carbohydrate metabolism	(Hyeon et al., 2012)
AsnC	Activate asnA, a gene involved in the synthesis of asparagine	(Thaw et al., 2006)
LrgB	Controlling hydrolase activity	(Groicher et al., 2000)
LysR	LysR family proteins control a diverse set of genes that are mainly involved in bacterial virulence, metabolism, quorum sensing, and motility	(Maddocks and Oyston, 2008)
DeoR	DeoR family of transcriptional regulators contains proteins that negatively control genes involved in carbohydrate metabolism	(Elgrably-Weiss et al., 2006)
WrbA	WrbA flavoproteins have been documented to act as RpoS-dependent stationary phase proteins	(Yang et al., 1993; Lacour and Landini, 2004)

3.4. Endophyte community variation drivers

A holobiont is the assemblage of a host, its microbiome, and any other organism that contributes to the functioning of the host and forms an ecological niche through mutual interconnections (Vandenkoornhuyse et al., 2015). The evolutionary history of the holobiont may be shaped significantly by the response of microorganisms to environmental changes, as well as the host organism itself (Occhipinti, 2013; Vandenkoornhuyse et al., 2015; Rosenberg and Zilber-Rosenberg, 2016; Hassani et al., 2018). Endophytic bacteria are typically defined as individual microorganisms rather than communities, whereas plants are influenced mainly by microbial communities. A microbial community can be defined as an assemblage of co-occurring and potentially interacting microbes present

in a defined habitat in space and time (Compant et al., 2010b). In this section, we review the effects of biotic and abiotic stresses on these communities and their effects on plants.

During the colonization of the endosphere, osmotic pressure, oxygen availability, carbon sources, and pH of the environment change, and bacteria must quickly adapt (Singh et al., 2021). Soil type and plant development stage appear to be the most important drivers of bacterial endophytic communities, whose composition is influenced by biotic factors and abiotic factors (Figure I-4, Table I-2). Biotic factors include (1) plant genotype (Hardoim et al., 2011; Yu et al., 2015; Ding and Melcher, 2016), developmental, and physiological stage (Jin et al., 2014; Yang et al., 2017); (2) composition of the root exudate and secondary metabolites (Aulakh et al., 2001; Shaw et al., 2006; Zhang et al., 2006; Dennis et al., 2010; Philippot et al., 2013; Vandenkoornhuyse et al., 2015; Henning et al., 2016; Musilova et al., 2016; Sasse et al., 2018); (3) biotic pollination; and (4) microbe-microbe interactions (Vandenkoornhuyse et al., 2015; Rosenberg and Zilber-Rosenberg, 2016).

Plant genotype, developmental and physiological stage—Plant genotype is one of the main factors. Research on bacterial endophyte communities is facilitated by high-throughput sequencing techniques, especially for root and phyllosphere microbiota. Next-generation sequencing (NGS) has revealed differences in the taxonomic composition of phyllosphere bacterial endophyte communities depending on the nature of the host plant species. Therefore, the core seed microbiome depends on the host genotype (Saikkonen et al., 2012). In addition to this genotypic effect, different plant organs harbor distinctive bacterial endophytes, suggesting that plants play a role in structuring endophytic communities (Jin et al., 2014; Yang et al., 2017). These variations in bacterial endophyte communities can also be explained by different environmental sources or by their ability to colonize different niches *in planta*. The examples are listed in Table I-2.

Root exudation—Plants can control variations in bacterial endophyte communities through root exudation. Plant exudate photosynthesis-derived compounds in the soil control the composition of microbial communities (Dennis et al., 2010; Philippot et al., 2013). Root exudate composition changes with plant species, cultivars, and developmental stages (Aulakh et al., 2001). For example, variation in phenolic compounds drives interactions between bacteria and plants (Shaw et al., 2006). Beneficial microbes are drawn to the root exudates because of their metabolites. Rhizospheric organisms use carbohydrates, lipids, amino acids, phenolics, phytosiderophores, and flavonoids as carbon sources (Badri and Vivanco, 2009). These metabolites modulate gene expression patterns in microorganisms to modify bacteria-host interactions (Mark et al., 2005; Yi et al., 2017). Due to the limited number of stud-

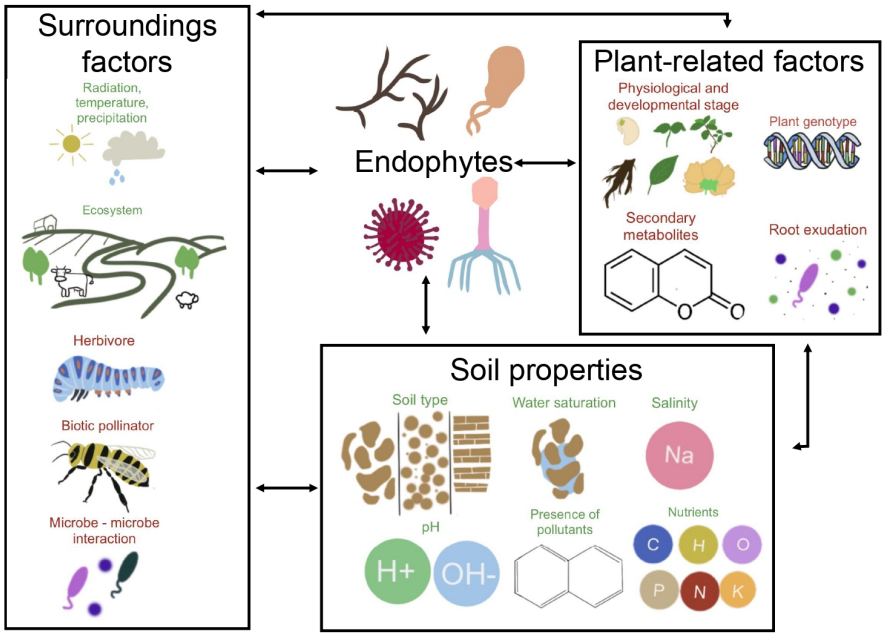

Figure I-4: Drivers of endophyte community variation, categorized into biotic (red) and abiotic (green) factors.
These drivers are further divided into three main categories: surroundings factors such as radiation, temperature, precipitation, ecosystem, herbivores, biotic pollinator or microbe-microbe interaction; plant-related factors such as physiological and developmental stage, plant genotype, secondary metabolites or root exudation; and soil properties such as the presence of pollutants, soil type, water saturation, nutrient availability, pH or salinity. Understanding the interplay between these drivers is crucial for comprehending the dynamics of endophyte communities and their responses to different ecological conditions

ies focusing on its impact on the composition of the bacterial endophyte community, the mechanisms underlying this influence are still poorly understood. Examples of the effects of root exudation on endophytic communities are listed in Table I-2

Plant secondary metabolites—Plant secondary metabolites (PSM), such as luteolin, quercetin, daidzein, and steviol glycosides (Papik et al., 2020), are another one of the most important factors influencing the structure and function of the endophyte community (Musilova et al., 2016; Sasse et al., 2018). PSM are a potential carbon source for rhizosphere and endophyte communities. They exhibit antimicrobial activity and induce the selection of microorganisms in the rhizosphere (Musilova et al., 2016). Indeed, plants achieve a delicate balance between attracting beneficial bacteria and deterring pathogens through sophisticated regulation of exudates and secondary metabolites. This

balance is maintained through spatial and temporal control of compound release, signaling pathways, and interactions with the plant microbiome (Bais et al., 2006). This dynamic system allows plants to foster beneficial relationships while protecting themselves from harmful microorganisms (Berendsen et al., 2012). For example, the more PSM are present in the rhizosphere, the higher the probability of microorganisms being capable of degrading pollutants because PSM can serve as primary substrates and/or enzyme inducers for the growth of microorganisms (Singer et al., 2003, 2004). Secondary metabolites can also influence gene expression, modulate host immunity, alter primary and secondary metabolism, and morphology of the host plant, which can result in plant growth promotion, increased stress tolerance, or reduced rates of herbivory during endophyte colonization (Zhang et al., 2006; Henning et al., 2016). Microorganisms found in the soil are primarily influenced by the rhizosphere, which constitutes the first plant-influenced habitat they encounter. Plant metabolism profoundly affects the thin layer of soil surrounding the roots through the release of oxygen and secretion of exudates, including carbon-rich molecules and antimicrobial compounds. Consequently, the rhizospheres between roots and soil are highly dynamic environments, resulting in differentiated microbial rhizosphere and endosphere communities (Vandenkoornhuyse et al., 2015).

Microbe-microbe interactions—Microbe-microbe interactions play an important role in endophyte variation. Interspecies interactions can affect the diversity and productivity of communities. Even if often described to be rare, positive interactions between microorganisms of soil microbiome often occur in coculture primarily as parasistisms (18%), commensalisms (12%) and mutualisms (5%); (Kehe et al., 2021). Microorganisms and host plants form a mutually inclusive ecosystem, which has recently been discussed regarding their mutual interconnections (Ochipinti, 2013; Vandenkoornhuyse et al., 2015). Endophytic bacteria can antagonize phytopathogens (Hu et al., 2009; Furnkranz et al., 2012; Khalaf and Raizada, 2018; Zhao et al., 2018). Fungi and bacteria can interact in the endosphere, which is crucial for the development of endophytic communities. Identifying the major endophytic taxa of certain crops and clarifying their unique roles in plants would contribute significantly to developing the aforementioned agricultural applications of endophytes and deepen our knowledge of the microbial composition in the endosphere. Some examples of microbe-microbe interactions are listed in Table I-2.

Abiotic factors influencing endophytic communities include (1) radiation and temperature (Redman et al., 2002; Ju et al., 2006; Nissinen et al., 2012); (2) precipitation and moisture (Rolli et al., 2015; Ullah et al., 2019a); (3) soil type (Buyer et al., 1999; Hinsinger et al., 2009; Polivkova et al., 2018); (4) pH (Hayman and Tavares, 1985; Wang et al., 1993; Anyango et al., 1995; Lafay and Burdon, 1998; Cornelissen et al.,

2011); (5) nutrients (Hermans et al., 2017); and (6) presence of pollutants (Vergani et al., 2017; Yergeau et al., 2018).

Radiation/temperature—Radiation and temperature affect endophytic communities, mostly by influencing host plant colonization (Redman et al., 2002; Ju et al., 2006; Nissinen et al., 2012). An important increase in temperature decreases shoot endophytic density because of reduced endophytic colonization (Pillay and Nowak, 1997). In contrast, a slight increase in temperature (+ 5°C) in temperate forests results in higher endophytic bacterial population (Rahman et al., 2021). It has been shown that climate change treatments, represented by elevated temperatures and/or increased CO_2 concentrations shift leaf-associated bacterial communities (Ren et al., 2015) and could impact bacterial functions such N_2 fixation (Nissinen et al., 2012; Rahman et al., 2021) (Table I-3).

Water and oxygen availability—precipitation, and soil moisture are also important factors affecting endophyte diversity. One common perturbation is drought stress, which impacts root bacterial communities directly by changing moisture availability and indirectly by altering soil chemistry and plant phenotypes. Many putative causes of endophytic bacterial community shifts have been hypothesized (Table I-3). First, naturally selected bacteria are directly resistant to drought stress. Some physiological mechanisms improve drought tolerance, including sporulation and thick cell walls, which are primarily found in bacteria belonging to Gram-positive phyla, such as Actinobacteria and Bacilli (Cherif et al., 2015; Tocheva et al., 2016). Differences in survival could also be explained by the ability to produce and accumulate osmolytes, such as amino acids (proline, glutamine, and glycine betaine) and carbohydrates (trehalose and ectoine). Gram-negative bacteria produce osmolytes only when they are constitutively produced (Schimel et al., 2007). Second, slow-growing microbes could be selected according to the nutrient-poor (less labile organic carbon and nitrogen) but oxygen-rich characteristics of drought environments. These characteristics favor oligotrophic bacteria with high metabolic activity, mostly Gram-positive bacteria (Yuste et al., 2014; Hartmann et al., 2017). The third hypothesis is that drought stress may induce the production of various compounds by bacteria, thereby affecting community stability. A high antibiotic content was found in drought environments, probably produced by drought stress-tolerant bacteria that outcompete other bacteria for limited resources (Bouskill et al., 2016). Taxa selected by drought stress and plants can be in the rhizosphere or endosphere and improve drought stress resistance in plants by maintaining host functions and fitness (Rolli et al., 2015; Ullah et al., 2019a). Several studies have reported a correlation between drought stress resistance in plants and a shift in their microbiome in response to stress (Naylor et al., 2017; Santos-Medellín et al., 2017; Fitzpatrick et al., 2018a). Flooding also affects endophytic communities, favoring the growth of anaerobic and aerotolerant bacteria. In

this environment, endophytic bacteria may play an essential role in fixing nitrogen as reported in rice (Ferrando and Fernández Scavino, 2015). Indeed, as oxygen irreversibly inhibits nitrogenase, N_2 fixers are favored in oxygen-poor environment (Smercina et al., 2019) (Table I-3).

Soil type—Soil type is described by Hinsinger et al. (2009) as one of the major abiotic drivers of endophyte community diversity. Multiple studies have shown that soil type strongly influence the endophytic bacterial and fungal communities (Buyer et al., 1999; Polivkova et al., 2018; Hou et al., 2019; Adeleke et al., 2022). Soil fertility, physicochemical properties, pH, porosity, water and oxygen content and soil organic carbon are interlinked to influence soil microbial density and activity (Rahman et al., 2021). The primary features of the soil, such as water-holding capacity, cation-exchange capacity, hydraulic conductivity, oxygen diffusion and humidity directly or indirectly influence soil bacterial communities and, indirectly influence endophytic bacterial communities. The relationship between the soil characteristics and bacterial taxonomic groups reflects the ecological functions of these organisms (Hermans et al., 2017). For example, soil with a high clay content has smaller interparticle spaces and, therefore, a lower oxygen content, which favors nitrogen-fixing bacteria.

pH—pH has been shown to affect microbial communities in the rhizosphere, but its impact also extends to the endosphere (Hayman and Tavares, 1985; Wang et al., 1993; Anyango et al., 1995; Lafay and Burdon, 1998; Cornelissen et al., 2011). A slight change in pH promotes the growth of more adapted species by lowering the original community (Rahman et al., 2021) (Table I-3).

Pollutants—The presence of pollutants in the soil selects microorganisms capable of using and/or degrading them as sources of carbon and energy (Vergani et al., 2017; Yergeau et al., 2018). Heavy metals, naturally present in soils but also used in fertilizers, pesticides, and wastewater irrigation, explained a portion of the variability in the abundance of bacterial phyla and classes (Table I-3).

Nutrients—Nutrients are essential for bacterial survival. The carbon-nitrogen ratio and the level of P may influence soil bacterial populations (Hermans et al., 2017).

Table I-2: Examples of biotic factors driving variation in endophytic communities.

Biotic factors	Description	Plant species, endophyte	Reference
Plant genotype	Influences of plant species on leaf endophytic bacterial communities of non-cultivated plants	*Ambrosia psilostachya* DC., *Asclepias viridis* Walt., *Panicum virgatum* L., *Sorghastrum nutans* (L.) Nash and *Ruellia humilis* Nutt	(Ding and Melcher, 2016)
	Determination of the different bacterial communities' composition across cultivars by rice genotype	*Oryza sativa* subspecies indica, japonica, and aromatica	(Hardoim et al., 2011)
	Conservation of a core microbiota in maize seeds across evolution with variations in relation to plant host phylogeny from teosinte to corn	Zea mays	(Johnston-Monje and Raizada, 2011)
Root exudation	Attraction of rhizobial endophytes and *Serratia* sp. thanks to flavonoids during colonization of rice roots	*Oryza sativa*, Rhizobial endophytes, Serratia sp.	(Balachandar et al., 2006)
	Attraction of endophytes thanks to exudates composed of citric acid and oxalate from the tricarboxylic acid flux and promotion of biofilms	*Cucumis sativus*, *Bacillus amyloliquefaciens* SQR9	(Zhang et al., 2014)
Microbe - microbe interaction	Mutualistic interactions between salt-tolerant endophytes enhancing the fitness of the plant	*Bacillus subtilis*, *Mesorhizobium cicero*, *M. ciceri*	(Egamberdieva et al., 2017)
	Application of keystone species theory to plant-associated microbiota for the first time	Inoculation of *Enterobacter* sp. E5, *Kosakonia* sp. S1 and *Klebsiella* sp. Kb on banana endosphere increase resistance to the Fusarium wild disease	(Liu et al., 2019; Macedo-Raygoza et al., 2019; Zhang et al., 2019b)

Table I-3: Examples of abiotic factors driving variation in endophytic communities.

Abiotic factors	Description	Plant species, endophyte	Reference
Radiation and temperature	Elevated temperatures and CO_2 concentration impacted the leaf-associated bacterial communities of rice	*Oryza sativa*, *Enterobacteriaceae*, *Xanthomonadaceae*	(Ren et al., 2015)
	Temperature affects the key enzymes used to fix N_2 by bacteria	*Azotobacter chroococcum*	(Miller and Eady, 1988)
	Many psychrotolerant endophytic bacteria were found in plant tissues of Arctic plants species	Endophytes of *Oxyria digyna*, *Diapensia lapponica* and *Juncus trifidus*	(Nissinen et al., 2012)
Water and oxygen availability—drought	Gram-positive phyla are favored thanks to their thick cell and spores	Actinobacteria and Bacilli	(Schimel et al., 2007; Tocheva et al., 2016)
	Plants modulate their cell wall components in response to drought with components that are usable by Actinobacteria	Actinobacteria	(Naylor et al., 2017)

Table I-3 (continued) : Examples of abiotic factors driving variation in endophytic communities.

Abiotic factors	Description	Plant species, endophyte	Reference
	Enrichment of Actinobacteria (*Streptomyces* genera) in the endosphere was related to drought resistance in plants	Actinobacteria, *Streptomyces*	(Fitzpatrick et al., 2018a)
	Enrichment of Actinobacteria in roots of *Poaceae* and rice plants	Poaceae, *Oryza sativa*, Actinobacteria	(Naylor et al., 2017; Santos-Medellín et al., 2017)
Water and oxygen availability—flooding	Depletion of Actinobacteria, Firmicutes, and Alphaproteobacteria phyla and enrichment of Gammaproteobacteria phylum after flooding of wheat phyllosphere microbiota	*Triticum aestivum*, Actinobacteria, Firmicutes, Alphaproteobacteria, Gammaproteobacteria phyla	(Francioli et al., 2022)
	Dominance of Gammaproteobacteria and Betaproteobacteria in a diazotrophic community of rice roots submitted to flooding stress	*Oryza sativa*, Gammaproteobacteria, and Betaproteobacteria phyla	(Ferrando and Fernández Scavino, 2015)

Table I-3 (continued) : Examples of abiotic factors driving variation in endophytic communities.

Abiotic factors	Description	Plant species, endophyte	Reference
pH	Abundance of members of *Pirellulaceae* is favored in neutral soil pH	*Pirellulaceae*	(Hermans et al., 2017)
	Pseudomonas oryzihabitans and *Rhizobium radiobacter* are favored in neutral pH soil, while in low-pH soil, *Enterobacter*-like and *Dyella ginsengisoli* are dominant	*Oryza sativa*, *Pseudomonas oryzihabitans*, *Rhizobium radiobacter*, *Enterobacter*-like, *Dyella ginsengisoli*	(Sessitsch et al., 2012)
	Acidic soil is a major driver of diazotroph communities' assembly	In qinghai-tibetan plateau plant, *Bradyrhizobium* and *Mesorhizobium* had different pH-relative abundance patterns	(Wang et al., 2017)
Pollutants	Increase of Proteobacteria phylum after graphene and graphene oxides application	Proteobacteria phylum	(Rong et al., 2017)
	Firmicutes and Actinobacteria phyla colonize heavy metal polluted soils	Firmicutes and Actinobacteria phyla	(Pires et al., 2017)
Nutrients	The carbon-nitrogen ratio influence soil bacterial population	*Gaiellaceae*, *Bradyrhizobium*	(Hermans et al., 2017)

4. Conclusion and future perspectives

This review aims to establish a refined and enhanced understanding of endophytes and endophytomes, considering the rapid technological advancements that have deepened our understanding of this concept. Additionally, we extensively examined the profile, colonization pathway, and various drivers responsible for the diversity and variation observed in the bacterial endophytome.

The urgent need for sustainable agriculture arises from the imperative to ensure global food security, mitigate environmental degradation, and build resilience in the face of climate change. Embracing sustainable practices is not merely an option but an essential strategy for safeguarding the well-being of present and future generations (Espinosa-Leal et al., 2018; Raza et al., 2019; Qaim, 2020; Delitte et al., 2021). The colonization of plants, animals, and humans by countless microorganisms has sparked a significant shift in the study of the effects of these microorganisms on the biology and well-being of their hosts (Drew et al., 2021). Numerous cases of microbial evolution causing transition across the 'parasite-mutualist continuum' have been reported both in human and plant health and will further emerge through research. For example, a healthy gut microbiome is now considered crucial for human growth, development, and immune system establishment, underscoring the importance of maintaining balance for optimal health (Kartjito et al., 2023). Recent data show that early-life gut microbiome development can protect against diseases linked to an imbalanced gut microbiome in later stages of life (Barone et al., 2023; Sabino et al., 2023). These findings support the idea that targeted therapies can restore the gut microbiome during infancy, potentially promoting long-term infant health (Charbonneau et al., 2017). Thus, modifying early colonization or addressing early-life gut dysbiosis may be strategies for enhancing well-being (Kapourchali and Cresci, 2020). The importance of microbiota in animal health has also been demonstrated in numerous studies, for example, in honeybees, where the genetic modification of a core gut bacterium improves resistance to viral infection (Leonard et al., 2020). In the future, engineering a microbiome or symbiont community via direct genetic modification of key transition loci in microbiome members could be used to improve human health. Future therapeutic and prophylactic modalities may be used to treat cancer and obesity (Sekirov et al., 2010).

In plant health, endophytes play a crucial role in alleviating both abiotic and biotic stresses. They enhance plant tolerance to adverse environmental conditions such as drought, salinity, and extreme temperatures. They stimulate the production of stress-related compounds and regulate plant hormone levels, thereby improving water and nutrient uptake efficiencies. Additionally, endophytes promote root development

and enhance plant defense mechanisms against abiotic stressors (Khare et al., 2018; Ullah et al., 2019a; Nadarajah, 2020; Singh et al., 2022). In response to biotic stress, endophytes shield against pathogens and herbivores by producing antimicrobial compounds, inducing systemic resistance, and activating plant defense pathways. These symbiotic microorganisms confer resistance to a wide range of pathogens, including bacteria, fungi, and nematodes, thereby ensuring plant health and productivity in the face of biotic challenges (Hardoim et al., 2008; Bulgarelli et al., 2013; Compant et al., 2019; Papik et al., 2020; Oukala et al., 2021). Overall, endophytes hold great promise for enhancing plant resilience and mitigating the negative impacts of abiotic and biotic stresses.

Drawing parallel to the human microbiota, there is a growing trend toward exploring endophytes in a similar manner. However, using a definition that targets only beneficial endophytes has prevented research from advancing as quickly as in the medical field. Moreover, studies of endophytes have been plagued by several limitations: (1) properties are studied in a single species or within closely related genotypes, but rarely across a taxonomically wide range of species; (2) the environmental conditions in which plant-endophyte interactions are investigated are often similar; (3) consortia are not often explored and bacterial and fungal endophytes are often considered separately (Elasri et al., 2001; Andreote et al., 2009; Hardoim et al., 2015). Consequently, our understanding of the modes of action of endophytes and their use in agriculture is limited.

The multiple interactions between endophytome, plants and environmental factors highlighted in this review reveals that endophytes need to be studied in terms of their endophytome as a community of microorganisms that share a common space. Using new technologies available in metagenomics, we argue that the dynamics of evolution of endophytic communities in plants during plant development and in response to biotic and abiotic stresses need to be explored using metatranscriptomics, metaproteomics, and metabolomics. Until now, the adoption of these technologies has been limited owing to their high cost and the complexity of the experimental and analytical procedures involved. However, the rapid evolution of technologies and price and cost decrease of these technologies promise for providing a more comprehensive perspective of the microbiome (Liu et al., 2021). This knowledge will allow the underlying mechanisms to be elucidated and products derived from well-adapted endophytes to be developed. Understanding the intricacies of endophytic communities can pave the way for sustainable agricultural practices.

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CHAPTER II. Role of endophytic bacteria in increasing plant drought stress tolerance

1. Introduction

The cumulative impact of human activities, particularly driven by the Industrial Revolution, has resulted in a significant increase in greenhouse gas emissions, consequently contributing to global warming. This phenomenon is characterized by a rise in average global temperatures, leading to a cascade of environmental consequences. One notable consequence is the heightened frequency and intensity of drought events, exacerbating water scarcity in various regions (Lobell and Gourdji, 2012; Ullah et al., 2019a). This, in turn, poses a serious threat to global food security, as agriculture heavily relies on adequate water availability.

In response to these challenges, innovative solutions are being explored to enhance the adaptability of agriculture to a changing climate. One such approach involves the cultivation of resilient crop varieties capable of withstanding the fluctuating environmental conditions associated with climate change. These specially bred varieties offer a proactive means of countering the detrimental effects on crop yields and securing food production in the long term. However, the development of these resilient crop varieties through selective breeding is a time-consuming process (Cattivelli et al., 2008).

The utilization of microorganisms has emerged as a sustainable alternative to traditional agricultural practices. Microorganisms, including bacteria and fungi, play pivotal roles in soil health and plant-microbe

interactions (Khan et al., 2009). In sustainable agriculture, these microorganisms are harnessed for their ability to promote plant growth, improve nutrient availability, and enhance the overall health of the agroecosystem. One notable aspect of this approach is the use of endophytes (Cocq et al., 2017). These microorganisms including bacteria, fungi, archaea, viruses, and other microbes, such as protists, protozoa, or archaebacteria, inhabit plants as obligatory or facultative organisms and are vertically or horizontally transmitted (Lengrand et al., 2024). A better knowledge of positive interaction between plants and endophytes can help to improve plant resilience to drought stress and advance sustainable development goals.

2. How can endophytic bacteria increase plant drought stress tolerance?

Plants have developed adaptations to drought stress tolerance including mechanisms of drought avoidance, tolerance, and recovery. Stress avoidance is realized through an improved water uptake thanks to an extensive root system, reduced transpiration losses due to stomatal closure, and increased water storage due to enhanced solutes accumulation (Malinowski and Belesky, 2000). Drought tolerance is mainly achieved thanks to the accumulation and translocation of assimilates, osmotic adjustment, and maintenance of cell wall elasticity. Following a drought stress event, the recovery of the plant depends mostly on the decrease of ROS content, based on enzyme activities such as catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO) and ascorbate peroxydase (APX). Indeed, the role of CAT, POX, PPO and APX may be associated in a better post-drought recovery as shown in a study with blue grass (Xu et al., 2011). Nevertheless, the mechanisms implemented by plants are frequently insufficient to guarantee their survival and microorganisms can augment their tolerance, helping in this regard.

Endophytic bacteria were found to increase plant tolerance to drought stress in numerous studies (Byregowda et al., 2022; Calvo-Polanco et al., 2016; Fua et al., 2021) and were identified to perform better than endophytic fungi (Tufail et al., 2022).

Diverse strategies are used to improve plant drought stress tolerance: (1) secretion of phytohormones such as abscisic acid (ABA), indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylic acid (ACC) -deaminase, and volatiles that impact plant hormonal balance, (2) production of exopolysaccharides (EPS) and osmolytes, such as proline and trehalose, that influence osmotic potential and relative water content, (3) secretion of antioxydants (Malinowski and Belesky, 2000; Ullah et al.,

2019b) and finally (4) production of molecules that promote plant nutrition. In most cases, multiple mechanisms are involved following inoculation with endophytic bacteria (Table II-1).

Bacteria possess the capability to influence the hormonal balance of plants by producing various plant hormones such as IAA, nitric oxide (NO), and ACC deaminase. IAA, known as an auxin, plays a crucial role in activating H^+-ATPase, which enhances root length and density, ultimately increasing the volume of soil explored by roots (Comas et al., 2013; Creus et al., 2005). For example, *Azospirillum brasilense* production of NO and IAA has been shown to promote lateral root and hair development in tomatoes, particularly under drought stress conditions (Creus et al., 2005).

In parallel, cytokinins, that help plants cope with drought stress by maintaining cellular functions and delaying senescence are also produced by bacterial endophytes (Bhore et al., 2010). For instance, inoculation of lettuce with cytokinin producing bacteria enhance lettuce growth in drying soil by increasing accumulation of shoot mass and shortened roots (Arkhipova et al., 2007).

The synthesis of ACC deaminase by bacteria triggers a reduction in ethylene (ET) levels, consequently also mitigating stress in plants (Ibort et al., 2018; Mayak et al., 2004). For example, tomato plants inoculated with *Streptomyces* sp. exhibited reduced expression of stress-responsive genes *WRKY70* and *ERF1*, leading to increased yield, leaf relative water content, and total sugar content (Abbasi et al., 2020). These findings suggest that bacterial inoculation alters the oxidative status of plants, possibly through the hormonal pathway. Indeed, in the study of Kumar et al. (2018), the application of *B. subtilis* and *Pseudomonas* sp. on tomato plants resulted in reduced oxidative stress, as evidenced by decreased activities of total polyphenol and enzymes CAT, POD, and PPO. This reduction was accompanied by an increase in salicylic acid (SA) and gibberellin (GB) content and a downregulation of stress-responsive ABA.

Improvement of the osmotic potential plays also a major role in stress avoidance and is frequently accompanied by an activation of the ROS-scavenging system. For example, *B. subtilis*, *B. thuringiensis* and *B. megaterium* have been shown to mitigate drought stress in Chickpea (*Cicer arietinum* L.) leading to increased leaf proline content, elevated activities of antioxidant enzymes (CAT, APOX, POD, and SOD), and enhanced accumulations of solutes such as riboflavin, L-asparagine, aspartate, glycerol, nicotinamide, and 3-hydroxy-3-methyglutarate (Khan et al., 2019). Similarly, the bacterial endophyte *Burkholderia phytofirmans* strain PsJN enhances drought tolerance levels in wheat plants by improving ionic balance and antioxidant levels (Naveed et al., 2013).

Numerous endophytes exhibit the capacity to fix nitrogen, solubilize phosphate and produce siderophores. For example, two *Pseudomonas*, *P. frederiksbergensis* and *P. brassicacearum*, isolated from roots of date palm and selected for their ability to solubilize phosphate and fix nitrogen, increased palm biomass under drought stress when applied to roots (Cherif et al., 2015). Improvement of morphological and biochemical parameters (stem and root length, plant fresh and dry weight, photosynthetic pigments, membrane damage) under normal and drought stress conditions was also observed after the application of *B. cereus* and *P. otitidis* on soybean. *In vitro* tests highlight that these strains possess some interesting plant growth promotion (PGP) traits such as IAA production, ACC deaminase, phosphate solubilization, ammonia production, hydrogen cyanide (HCN) production, and tolerance to drought (PEG 15%) (Dubey et al., 2021).

Table II-1: Positive effects of endophytic PGPB on tomato growth under drought stress

Endophytes	**Effect on plant**	**References**
Achromobacter piechaudii ARV8	Reduce ethylene production and increase of fresh and dry weight	(Mayak et al., 2004)
Azospirillum brasilense	Induce IAA pathway which enhanced lateral rooting and root hair development	(Creus et al., 2005)
Bacillus amyloliquefaciens CBa_RA37 and *B. megaterium* RR10	Change oxidative status, stomatal and photosystem II functioning, internal leaf temperature and relative water content. Increase activity of oxidative stress enzymes	(Eke et al., 2019)
B. megaterium or *Enterobacter* sp. strain C7	Reduce expression of ethylene-related genes	(Ibort et al., 2018)
B. subtilis and *Pseudomonas* sp.	Increase plant biomass, chlorophyll content and water potential. Reduce electrolytic leak, oxidative stress through lessened activities of total polyphenol and enzymes CAT, POX, PPO. Increase salicylic acid and gibberellin and reduce expression of stress-responsive ABA genes	(Kumar et al., 2018)

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Table II-1 continued from previous page

Endophytes	Effect on plant	References
Microbacterium oxydans	Increase drought stress tolerance, reduce ABA and jasmonic acid (JA) production	(Siraj et al., 2022)
Pseudomonas sp. and *Bacillus* sp.	Increase viability and vigor of tomato seed	(Fua et al., 2021)
Streptomyces sp.	Increase yield, leaf relative water content (RWC), proline and total sugar content. Increase APX activity and decrease CAT and glutathion peroxidase (GPX) activities. Reduce the expression of *ERF1* and *WRKY70* genes	(Abbasi et al., 2020)
Streptomyces globisporus SP6C4	Enhance tolerance to drought and salinity stresses in tomatoes when colonizing the rhizosphere and root endosphere	(Kim and Kwak, 2023)
Sphingomonas sp.	Decrease lipid peroxidation and EPS production	(Halo et al., 2015)
Variovorax paradoxus	Increase shoot dry weight, net photosynthesis, relative chlorophyll content and decrease proline content	(Calvo-Polanco et al., 2016)

CHAPTER III. Focus on seed-transmitted endophytic bacteria

1. Relevance of seed endophytic bacteria

Seeds serve a dual purpose in the plant life cycle, both initiating reproduction and sustaining the species, while also facilitating dispersal, adaptation, and persistence in new environments (Fenner and Thompson, 2005). Their involvement in key stages, from seed maturation to germination, underscores their pivotal role. Consequently, the microbial communities associated with these stages hold significant potential for supporting overall plant life (Bintarti et al., 2022). Indeed, the vulnerability of germinating seeds and seedlings to threats like drought and pathogens creates a crucial bottleneck in the plant's life cycle. Thus, microbial interactions during these stages significantly influence plant population dynamics in natural ecosystems and can determine crop success in agricultural systems (Nelson, 2004; Rodriguez et al., 2020).

Seed microbiomes represent a reservoir of microbial taxa that have co-evolved with plant hosts, possessing functions that could help in plant survival (Compant et al., 2010a; Hardoim et al., 2015, 2012; Johnston-Monje and Raizada, 2011). Indeed, vertical transmission ensures the continuity of microorganisms from parent plants to their offspring, reducing the potential pathogenicity of endophytes and encouraging beneficial endosymbiotic relationships (Barret et al., 2016; Cope-Selby et al., 2017; Hardoim et al., 2012; Rudgers et al., 2009; Shade et al., 2017). Several studies demonstrated that a diverse endophytic community is associated with seeds and has several important features such as production of phytohormones, enzymes, antimicrobial compounds, and secondary metabolites (Puente et al., 2009; Shahzad et al., 2018; Truyens

et al., 2015).

The portion of the bacterial endophyte population conserved through vertical transmission varies depending on factors such as plant species and environmental conditions. For instance, in the case of *Crotalaria pumila* grown on metal mine residues, three generations of seeds exhibited similar bacterial endophytic communities (Sanchez-Lopez et al., 2018). Similarly, in tomato plants, Bergna et al. (2018) showed that endophytes persist in seedlings for two generations and Moroenyane et al. (2021) highlighted that the seed microbial communities of soybean take priority over soil microbial communities during plant root and rhizosphere colonization (Santoyo et al., 2016; Shade et al., 2017). Conversely, other studies have shown that only a small fraction of the seed microbiome endures across generations, with a significant portion of the seedling microbiota originating from the surrounding soil (Rochefort et al., 2021; Rodriguez et al., 2020).

2. Seed bacterial endophytome composition

Endophytes can be transmitted to plant seeds through both vertical and horizontal pathways. Vertically, the transmission can occur asexually when seed-borne endophytic bacteria colonize flowers and migrate to the developing seed via the vascular system and intercellular spaces, utilizing structures such as the funiculus, chalaza, and micropyle. There is also a sexual route of transmission, where microorganisms are passed through male gametophyte, inside and on surface of pollen subsequently colonizing the embryo and the endosperm (Zasloff, 2017). Moreover, if a microbe is present in a reproductive cell (such as an egg cell or male gamete), it could potentially colonize the resulting embryo and endosperm (Malfanova et al., 2013; Truyens et al., 2015). This hypothesis is supported by findings that flowers, pollen, and ovaries harbor microbial species akin to those in seeds (Abdelfattah et al., 2021). Additionally, endophytes may also be horizontally transferred from the soil or other external vectors, contributing to the diversity and composition of seed microbiota (Bergna et al., 2018; Rodríguez et al., 2018; Mitter et al., 2017; Johnston-Monje and Raizada, 2011; Rodríguez et al., 2018).

Initially, seed-transmitted endophytic bacteria were studied through cultivation-dependent methods with typical members of genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Micrococcus*, *Pantoea*, and *Acinetobacter* (Truyens et al., 2015) and with a lot of spore-forming bacteria resistant to desiccation (Sobolev et al., 2013). Yet, due to specific habitat of seed endophytes, their cultivation is very challenging leaving the majority of this microbial community undiscovered. This limitation was overcome with new advances in sequencing. This approach reveals that the bacte-

rial microbiota found in seeds primarily consists of *Proteobacteria* being the most prevalent phylum, followed by *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* (Barret et al., 2015; Hardoim et al., 2012; Johnston-Monje and Raizada, 2011; War et al., 2023).

Seed microbiota exhibit remarkable diversity and variability, with richness ranging from a single taxon to thousands. As desiccated tissues, seeds primarily support bacterial endophytes that can withstand high osmotic pressures (Mano et al., 2006; Rana et al., 2020). The way in which the bacteria survive within the seed and their mode of nutrition, however, remain however still unknown. The diversity of bacterial species in seeds varies widely among different plant species, genotypes, and geographic locations, reflecting the influence of root exudates that attract specific microorganisms from the soil, thus shaping the endophytic communities (Rochefort et al., 2019; Zhang et al., 2019a; War et al., 2023; Compant et al., 2010a; Brader et al., 2017; Turner et al., 2013; Barret et al., 2015; Hardoim et al., 2015; Afzal et al., 2019). However, Guo et al. (2021) argues that the seed microbiome composition is not solely determined by plant genetics or local environmental factors. Overall, the seed microbiome's composition is complex, influenced by both the immediate environment and intrinsic host plant factors (Johnston-Monje and Raizada, 2011; Zhang et al., 2019a).

While plant species do impact the composition of seed microbiota, certain bacterial species appear to be prevalent across many plant types. For example, Simonin et al. (2022) identified dominant genera consistently found in the seeds of 50 different plant species, including *Pantoea agglomerans*, *Pseudomonas viridiflava*, and *Pseudomonas fluorescens*.

3. Evolution of bacteria present in seeds across plant structures

Endophytes are found in all parts of the seed, including the seed coat, the endosperm, and the embryo (Barret et al., 2015; Rodríguez et al., 2018) (Figure III-1). In the study of Glassner et al. (2018), scanning electron microscopy (SEM) images and fluorescence *in situ* hybridization (FISH) analysis highlighted the diverse distribution of bacterial communities within *Cucumis melo* seed tissues. Alphaproteobacteria were found on the seed coat and around the embryonic hypocotyl-root tissues, Betaproteobacteria and some Gammaproteobacteria were present in the outer seed coat, while Firmicutes and Actinobacteria predominantly inhabited the cotyledons and other embryonic tissues. Notably, embryo of oak acorns demonstrates even higher microbial diversity and richness compared to the pericarp (Abdelfattah et al., 2021). However, during

germination and emergence, microbial alpha diversity decreases due to an increase of relative abundance of certain bacterial taxa with rapid growth abilities (Barret et al., 2015; Cope-Selby et al., 2017)..

Distinct communities are transmitted to seedling's phyllosphere and roots suggesting niche specialization among microbes. The phyllosphere exhibits a diverse microbial community close to the microbiota of the embryo, while roots harbor a less diverse and distinct microbial community when grown in hydroponics or in soils deprived of bacterial communities (Hardoim et al., 2012; Abdelfattah et al., 2021; Faddetta et al., 2021). This migration pattern may be influenced by differences in the life-history traits of microorganisms in relation to anatomical and physiological properties of plants tissues (Abdelfattah et al., 2021). Additionally, the endophytome evolves during plant life according to the stage of development and environmental conditions (Barret et al., 2015). Certain bacteria are restricted to specific plant tissues while others are systematically distributed throughout the plant with a bacterial mobility that can be accompanied by the production of cell wall-degrading enzymes (Compant et al., 2005). Part of non-obligate endophytic bacteria may exit the roots to the rhizosphere, and some are able to exit leaves through stomatal apertures (Compant et al., 2005).

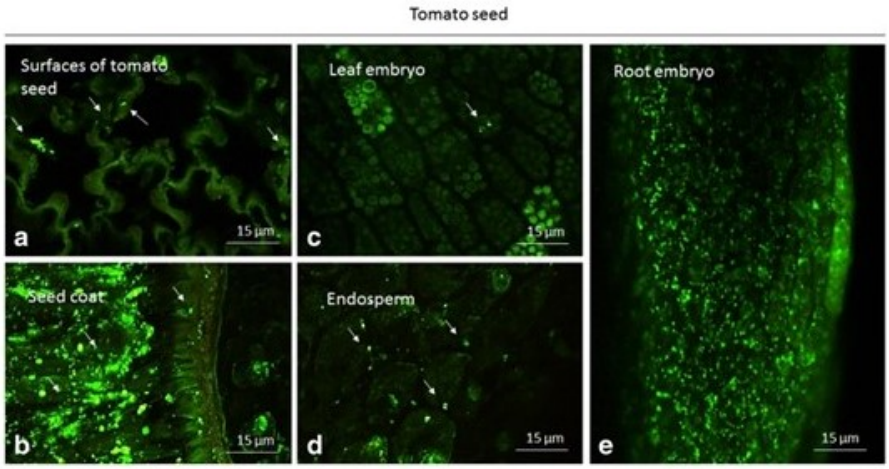

Figure III-1: Microphotographs of *S. lycopersicum* L. cv. Moneymaker seeds inoculated with fluorescent bacteria images from Rodríguez et al. (2018)

Presence of green fluorescent bacteria on surfaces of the seed, inside the endosperm, leaf, and root embryo after Syto9® staining and confocal laser scanning microscopy. Similar finding was obtained for inner tissues using surface sterilized seeds or not.

Bacterial endophytes primarily occupy intercellular spaces due to the abundance of carbohydrates, amino acids, and inorganic nutrients (Hardoim et al., 2015). However, intracellular colonization was also observed in *Vitis vinifera* L. cv, in shoot tips of banana, shoot meristem of

Scotch pine, seedlings roots of switchgrass, and micropropagated peach palm (Compant et al., 2005; Thomas and Shaik, 2020; de Almeida et al., 2009; Pirttilä et al., 2000; White et al., 2014; Thomas and Sekhar, 2014; Esposito-Polesi et al., 2017). This intracellular colonization is probably possible thanks to points of entry of roots hairs, such as in legume-rhizobium symbiosis, thanks to secretion of cell wall degrading enzymes, or through rhizophagy (Kandel et al., 2017; Paungfoo-Lonhienne et al., 2010; Prieto et al., 2011; White et al., 2014). However, little is known of how seed endophytic communities really colonize the plant as most of studies are realized in non-sterile conditions or even in soil, adding a bias in the repartition of these communities.

4. Seed-borne bacterial endophytes of *S. lycopersicum*

Among the various crops, tomato (*S. lycopersicum*) holds significant importance as a major horticultural crop. Despite its importance, knowledge about the tomato seed microbiota remains relatively limited.

The Table III-1 offer a perspective of the current knowledge about seed-transmitted endophytic bacteria of (*S. lycopersicum*). Cultivation-based methods have frequently identified directly in the seeds, a prevalence of *Bacillus* species, alongside *Micrococcus* sp. and *Paenibacillus* sp. (Sanchez-Lopez et al., 2018; Sharma et al., 2021). In contrast, next-generation sequencing (NGS) techniques have revealed a bacterial community predominantly composed of Proteobacteria, including families such as *Burkholderiaceae*, *Pseudomonadaceae*, *Comamonadaceae*, and *Moraxellaceae*, with additional presence of Actinobacteria, Bacteroidetes, and Firmicutes (Bergna et al., 2018; Thomas and Shaik, 2020; Sanchez-Lopez et al., 2018). Furthermore, cultivation-based studies on plantlets aged 7-8 weeks, grown *in vitro*, have demonstrated the presence of various bacteria, including *Enterobacter oryzendophyticus* and *Ralstonia pickettii* (Shaik and Thomas, 2019). Notably, Bergna et al. (2018) conducted a comparative analysis of seed and root endophytic communities under non-sterile conditions using 16S V4 sequencing. Their findings underscored a similar microbial diversity in both the seeds and the roots' endosphere, with a significant representation of Proteobacteria.

Table III-1: The composition of seed-transmitted bacterial endophytic communities of *S. lycopersicum*.

Cultivars of *S. lycopersicum*	**System of culture**	**Timing**	**Method of Identification**	**Bacterial Communities**	**References**
Cultivars "Arka Vikas" and "Arka Abha"	/	Study directly on seeds	V1V3 region of 16S rRNA sequencing	Gammaproteobacteria: Moraxellaceae with 66.7 and 62.3% in both cultivar and *Psychrobacter* as main genus followed by *Moraxellaceae*. Bacilli: Planococcaceae (*Planococcus* and *Planomicrobium*). Actinobacteria: Nocardiaceae (*Arthrobacter*, *Rhodococcus*). Bacteroidetes: *Gillisia*	(Thomas and Shaik, 2020)
Cultivars Elpida F1 and Silverio	/	Study directly on seeds	V1V3 region of 16S rRNA sequencing	Proteobacteria (18 and 65% according to the cultivar) with *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Shinella*, *Sphingobium*, *Rhizobium*, *Ensifer*, *Sinorhizobium*, *Achromobacter*, *Acidovorax*, *Pantoea*, *Pectobacterium*, *Serratia*. Actinobacteria (14.3% and 27.3%) with *Clavibacter*, *Corynebacterium*, *Micrococcus*, *Curtobacterium*, *Microbacterium*. Bacteroidetes (1,2 and 3%) with *Flavobacterium* and *Sphingobacteriu*m. Firmicutes (64 and 3%) with *Paenibacillus* and *Staphylococcus*	Lopez et al., 2018

Table III-1 (continued)

Cultivars of *S. lycopersicum*	**System of culture**	**Timing**	**Method of Identification**	**Bacterial Communities**	**References**
Cultivars Elpida F1 and Silverio	/	Study directly on seeds	Cultivation-based studies	*Micrococcus* sp., *Bacillus* sp.,*Sphingomonas* sp, *Brevundimonas* sp., *Paenibacillus* sp., *Jeotgalibacillus* sp, *Acinetobacter* sp., *Microbacterium* sp.,*Psychrobacillus* sp., *B. subtilis*	(Sanchez-Lopez et al., 2018)
Cultivars "Arka Vikas" and "Arka Abha"	*In vitro* on Murashige and Skoog medium	*In vitro* seedlings of 35 days	Cultivation-based studies	*Enterobacter oryzendophyticus*, *Ralstonia pickettii*, *Sphingomonas paucimobilis*, *Sphingobium yanoikuyae*, *Micrococcus aloeverae*,*Ralstonia mannitolilytica*, *Brachybacterium conglomeratum*, *Kocuria subflava*, *Micrococus aloevereae*, *Bacillus phocheonensis*	(Shaik and Thomas, 2019)
Cultivar Pusa Ruby and a local variety of Andhra Pradesh	Sterile, *in vitro*, germination on sterile filter paper	Seedlings of 9 days of germination	Cultivation-based studies	*Bacillus safensis*, *B. siamensis*, *B. australimaris*, *B. subtilis*, *B. nakamurai*, *B. zhangzhouensis*, *B. amyloliquefaciens*	(Sharma et al., 2021)

Table III-1 (continued)

Cultivars of *S. lycopersicum*	**System of culture**	**Timing**	**Method of Identification**	**Bacterial Communities**	**References**
Cultivars Moneymaker and Hildares F1	Sterile quartz-sand and diluvial sand	Plants of 85 days after sowing	16S sequencing - V4	Seeds (Proteobacteria 64 % : Burkholderiaceae(19%), Pseudomonadaceae(7%), Comamonadaceae (6%), Rhizobiales, Micrococcaceae. Actinobacteria (8%), Firmicute (9%), Bacteroidetes (7%). Roots endosphere (Proteobacteria 69% : Pseudomonadaceae (18%), Comamonadaceae (6%) and Rhizobiaceae (6%). Actinobacteria (8%). Firmicute 19% : Bacillaceae (6%) and Bacteroidetes (7%).	(Bergna et al., 2018)

CHAPTER IV. Interplay of humic substances and endophytic bacteria in plant health

1. Definition of humic substances

Humic substances (HS) represent the major pool of organic carbon at the earth's surface and contribute to many environmental processes, such as soil aggregate stabilization, plant nutrients supply, water-holding capacity, and carbon and nitrogen cycling (Piccolo, 1996). HS are supramolecular conformations composed of relatively small and heterogeneous molecules associated by weak bonds: dispersive hydrophobic bonds (van der Waals, π–π, and CH–π bondings) and hydrogen bonds. When the pH of the solution is lowered, hydrogen bonds become more important (Piccolo, 2002) and in solution, HS appear as large molecular size associations due to hydrophilic and hydrophobic domains that can be contiguous to or contained in each other.

According to their solubility, HS are classified into three categories. First, humic acids (HA) made by associations of predominantly hydrophobic compounds (polymethylenic chains, fatty acids, phenolic, and steroid compounds) (Figure IV-1). These molecules are soluble at neutral pH but insoluble when the pH is adjusted to 1-2 (Hayes, 2006). Second, fulvic acids (FA) composed of small hydrophilic molecules with strong acidic functional groups that allow the stabilization of the supramolecular structure under any pH (Figure IV-1). Finally, HS are also composed of humin, which is insoluble at all pH (Stevenson, 1994). Due to this particularity, only humic and fulvic fractions are found in commercial products and represent an important proportion of the biostim-

ulants market. In this chapter, we will collectively refer to HA and FA as humic substances (HS).

HS can be extracted from naturally humidified organic matter with sedimentary origin (mostly leonardite) or from organic substances obtained by composting organic materials such as plant or animal fresh residues (García et al., 2016).

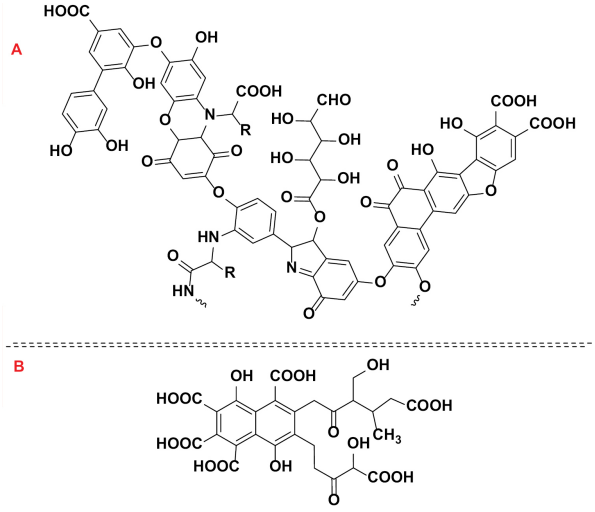

Figure IV-1: Hypothetical models of molecular structures of humic (A) and fulvic (B) acids (Wang and Mulligan, 2006)

2. Mechanisms of action of humic substances used as biostimulant

HS have gained significant attention for their role as biostimulants in agriculture, enhancing plant growth, nutrient uptake, and stress tolerance without being fertilizers, pesticides, or soil amendments in the traditional sense. Their biostimulant properties stem from their ability to improve soil structure, increase nutrient availability, and stimulate plant physiological processes.

The complex structure of HS complicates understanding their action mechanisms. Despite extensive studies on HS, their biostimulatory mechanisms remain elusive. Currently, the effects of HA and FA on plants are classified into indirect impacts on soil and microbial communities, and direct activation of hormonal and ROS pathways (Figure IV-2) (García et al., 2016).

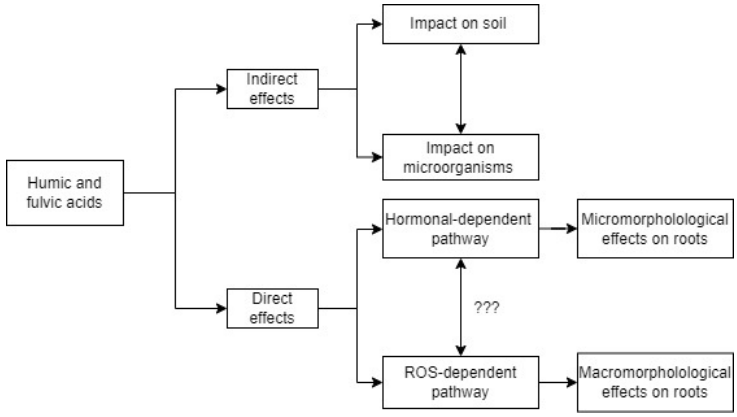

Figure IV-2: Main categories of biostimulatory activities of humic and fulvic acids (Adapted from García et al. (2016))

2.1. Indirect effects on plant metabolism

HS improves indirectly plant growth mainly by impacting the soil. Indeed, HA and FA favor soil structure being recalcitrant, hydrophobic molecules and constituents of stable and humidified soil organic matter (SOM) fractions. Their porous structure and hydrophobic regions facilitate the formation of aggregates and complexes with soil clays. Additionally, their carbon content bolsters aggregate stability and influences soil microorganisms, resulting in improved soil stability, aeration, and water retention (Piccolo, 2002).

Building on the structural benefits provided by HS, these molecules indirectly improve plant mineral nutrition in two ways: by enhancing soil cation exchange capacity (CEC) through their polyanionic structure and by forming soluble complexes with micronutrients to prevent leaching (Nardi et al., 2002; García et al., 2016). The polyanionic structure of HS increases the soil's CEC, which enhances the soil's ability to retain essential cations like potassium, calcium, and magnesium (Elkins and Nelson, 2002). This increased retention makes these nutrients more available to plant roots, thereby improving overall nutrient uptake (Nardi et al., 2002). Additionally, by forming soluble complexes with micronutrients, HS prevent these nutrients from being leached away by irrigation or rainfall, ensuring their availability for plant uptake (Cattani et al., 2009). The effects of HS on soil properties and plant growth are dose-dependent, with optimal benefits observed at specific concentrations (Calvo et al., 2014).

While plants can directly absorb cation-HS complexes, facilitating nutrient uptake, this is primarily limited to the smaller FA. FA are small enough to penetrate plant cell walls and enter the plant's vas-

cular system, directly enhancing nutrient uptake (Nardi et al., 2002). In contrast, larger molecules such as HA cannot be absorbed directly by plants. Instead, they interact with the cell walls, enhancing root permeability and nutrient absorption indirectly by improving soil structure and microbial activity around the root zone (Trevisan et al., 2010). This interaction helps create a more favorable environment for nutrient uptake and overall plant growth (Figure IV-3).

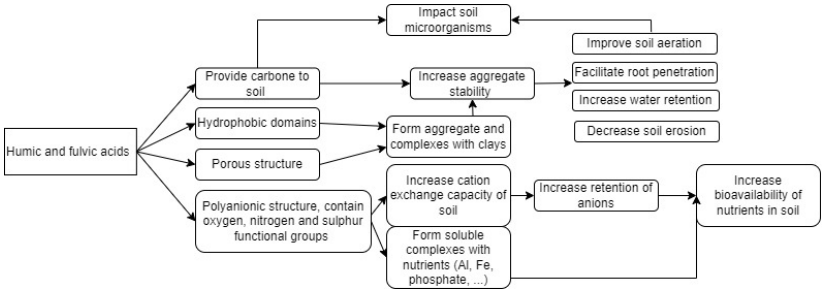

Figure IV-3: Indirect effects of humic and fulvic acids used as biostimulants
(Cattani et al., 2009; Elkins and Nelson, 2002; Magdoff and Weil, 2004; Piccolo, 2002; Wang and Mulligan, 2009)

2.2. Direct effects on plant metabolism - hormonal pathway

By conducting experiments in hydroponic systems, researchers have investigated the direct effects of HS on plants. The findings presented in Table IV-1 unveil a wide spectrum of outcomes arising from HS application to various crop species and under diverse stress conditions. These effects encompass enhancements such as improved yield and fruit firmness in strawberries (Farahi et al., 2013), enhanced shoot and root growth in tomato seedlings (Lulakis and Petsas, 1995), elevated net photosynthesis and fruit sugar content in tomatoes (Haghighi et al., 2010), and increased above-ground biomass in lettuce (Guilayn et al., 2020). Notably, maize exhibited improved nitrate metabolism and enzyme activity (Vaccaro et al., 2015), while tomato plants displayed stimulated growth and enhanced ion uptake (Adani et al., 1998). In rice plants subjected to osmotic stress, HS application mitigated oxidative stress and lipid peroxidation (Garcia et al., 2014), while strengthen the antioxidant defense system (García et al., 2012). This trend was similarly observed in soybean, where HS application upregulated the antioxidant defense system under osmotic stress (Matuszak-Slamani et al., 2022). Furthermore, in barley and strawberry, HS reversed symptoms induced by salinity, underscoring its role in fortifying plant resilience (Saidimoradi et al., 2019; Jarošová et al., 2016). Additionally, in cucumber, HS led to a significant

increase in shoot and root dry weight accompanied by heightened root hydraulic conductivity (Lpr) and plasma membrane (PM) H^+-ATPase activity in roots, as well as increased root IAA concentration (Olaetxea et al., 2019). Similarly, the application of HS and FA resulted in enhanced shoot growth, associated with notable increases in ABA root concentration and root hydraulic conductivity in cucumber (Olaetxea et al., 2015). To resume, major effects of HS application result in increasing of root surface area, enhancing absorption and translocation of nutrients and reduce oxydative stress (Canellas et al., 2015). These findings collectively highlight the multifaceted effects of HS on plant physiology and stress response in hydroponic environments.

Following all these observations, it is recognised that HS directly influence plant metabolism through hormonal and ROS signaling pathways. However, the precise event triggering the chain of interconnected molecular effects responsible for the positive impacts of HS remains unclear.

Three main hypotheses have been proposed to elucidate this phenomenon, with the first two leading to activation of the hormonal pathway and the third being ROS-dependent (Figure IV-4). The first hypothesis suggests that HS include phytoregulators or plant hormones, primarily molecules similar to IAA, within their structure. These hormones may be released when HS come into contact with root exudates, allowing them to interact with hormone cell receptors and activate hormonal pathways (Canellas et al., 2002; Piccolo, 2002; Trevisan et al., 2010). This process is considered feasible for organic materials that have undergone composting or vermicomposting, as their relatively fresh state may still contain phytohormones. However, the likelihood of ancient sediments containing active phytohormones is diminished (Canellas et al., 2015; Trevisan et al., 2010).

A second hypothesis suggests that HS possess structural domains that share steric and electronic characteristics with auxin, potentially activating similar hormonal pathways in plants (Trevisan et al., 2010). However, this hypothesis appears less plausible given the significant size difference between auxin and structural units of HS, questioning the likelihood of such interaction (Calderon-Villalobos et al., 2010).

In both cases, the interaction between HS and roots surface leads to an activation of the auxin pathway. This activation has been proven since the auxin synthetic reporter *DR5::GUS* was activated by HS application and transcription of early auxin responsive gene *IAA19* was enhanced in *Arabidopsis* (Trevisan et al., 2010). This hormonal activation is interconnected with other pathways, affecting the metabolisms of NO, ET, and ABA, leading to alterations in hormone levels, gene expression, and the stimulation of various proton pumps: plasma mem-

Table IV-1: Effects of humic substances on crop species in hydroponics

Crop species	Stress	HS type	Effects	References
Strawberry	No	HA commercial soluble product; foliar spray at different doses under greenhouse	Enhanced yield (33%) and fruit firmness	(Farahi et al., 2013)
Tomato	No	HA and FA from compost at different doses, growth chamber	Doses–response curve for shoot and root growth of tomato seedlings; HA were more bioactive than FA	(Lulakis and Petsas, 1995)
Tomato	No	Different HA from forest soil mixed with nutrient solution	Enhanced net photosynthesis by 68–436% during the vegetative stages and increased fruit sugar content	(Haghighi and Silva, 2013)
Lettuce	No	Two digestates (sewage sludge and manure)	Aerial biomass increase averages ranged from 7 to 30%, but the results presented a high coefficient of variation (around 20%)	(Guilayn et al., 2020)
Maize	No	HS extracted from a volcanic soil	HS at low concentration (1 mg C. L^{-1}) positively influenced nitrate metabolism by increasing the content of soluble protein and amino acids synthesis. Stimulation of the activity and transcription of enzymes functioning in N assimilation and Krebs Cycle	(Vaccaro et al., 2015)

Table IV-1 (continued)

Crop species	Stress	HS type	Effects	References
Tomato	No	Two commercially-available products (CP-A prepared from peat and CP-B prepared from leonardite)	Both HA stimulated plants growth (root and shoot) and increase total ion uptake (N, P, Fe, Cu)	(Adani et al., 1998)
Rice	Osmotic stress (15% PEG)	Vermicompost HA (20 mg $C.L^{-1}$, 40 mg $C.L^{-1}$, and 80 mg $C.L^{-1}$)	Decrease H_2O_2 and lipid peroxidation. Mechanisms mediated by tonoplast aquaporins (*OsTIPs*). Plants under water stress and treated with HA maintained ABA levels similar to those of the control plants. HA exerted differential effects on the expression of aquaporin in both root and leaf tissues. Alkyl, carboxylic and carbonyl structures predominated in the agglomerated HA at the roots.	(Garcia et al., 2014)
Barley	Salt stress (NaCl)	HA	HA increased the content of organic metabolites (syringic acid, alanine, proline, ascorbic acid, glutathione, and phytochelatin). Salinity induced ROS formation, and HA reversed these symptoms	(Jarošová et al., 2016)
Strawberry	Salt stress	HA	Improved plant responses to salinity, reduced the leaf necrosis area caused by salinity. Salt tolerance index was improved by increasing the amount of RWC and the cell membrane stability index using the humic acid.	(Saidimoradi et al., 2019)

Table IV-1 (continued)

Crop species	Stress	HS type	Effects	References
Soybean	Osmotic stress	Molecular fractions of humic acids (HA): HA $< 30kDa$ and $HA > 30kDa$	$HA > 30kDa$ fraction better up-regulated the antioxidant defense system.	(Matuszak-Slamani et al., 2022)
Rice plants	No	HA	Positive influence of HAs on the growth of rice and its stimulation of the root system.	(da Silva et al., 2021b)
Rice plants	Osmotic stress	HA were extracted from vermicompost	HA induced POX activity leading to a reduction in H_2O_2 content and greater conservation of membrane permeability.	(García et al., 2012)
Cucumber	No	HA extracted from leonardite (100 mg/L of organic carbon)	A significant increase was observed in shoot and root dry weight after 72h of treatment. At the same time, Lpr, PM H^+-ATPase activity in roots and root IAA concentration were increased.	(Olaetxea et al., 2019)
Cucumber	No	HA and FA extracted from leonardite applied at 100 mg/L of organic carbon	Shoot growth enhancement associated with significant increases in ABA root concentration and root hydraulic conductivity.	(Olaetxea et al., 2015)

brane PM H^+-ATPase), vacuoles H^+-ATPase and H^+-pyrophosphatases activities (Mora et al., 2010; Quaggiotti, 2004; Zandonadi et al., 2007). In addition to direct stimulation of proton pumps, HS application overexpresses proteins that upregulate the tonoplast enzymes V-ATPase and H^+-ATPase. This proton efflux created an electrochemical gradient, enhancing the plant's ability to import nutrients and ions (Morsomme and Boutry, 2000; Rayle and Cleland, 1992; Nardi et al., 2002).

In parallel, acidification of the cell wall led to its loosening, increased cell division and proliferation, as well as proliferation of lateral roots emergence sites. All of this leads to an increase in root growth, weight, and absorption surface (Mora et al., 2012; Olaetxea et al., 2019; Trevisan et al., 2010; Zandonadi et al., 2007).

Furthermore, the acidification of the cell wall, driven by proton extrusion, activates pH-sensitive enzymes and proteins, which impacts primary metabolism, mainly carbon, nitrogen, and sulfur metabolism.

Nardi et al. (2007) showed that HS application increases activities of enzymes related to glycolysis and the tricarboxylic acid cycle. This treatment also raises carbon fixation gene expression in *Brassica napus*, resulting in larger and more numerous starch granules in chloroplasts (Jannin et al., 2012). Conversely, application of humic fractions on maize seedlings decreases starch content while increasing soluble sugar concentration (Nardi et al., 2007). Merlo et al. (1991) suggests that activities of invertases and sucrose synthase were stimulated by HS treatment in apical tissue, and inhibited in basal one.

The nitrogen metabolism in plants is also influenced by HS treatment at different levels, including uptake, assimilation, and translocation. Indeed, Quaggiotti (2004) and Jannin et al. (2012) observed an upregulation of nitrate transporters following HS treatment. However, no relationship between increased nitrate uptake and high/low affinity of NO_3- cell transporter to HS stimuli was found. This suggests that HS effect on NO_3- uptake is more a consequence of the creation of an electrochemical gradient that facilitates the H^+/NO_3- symport rather than a direct effect on NO_3- transporters (Nardi et al., 2000). Additionally, HS treatment has been observed to increase the translocation of nitrates from roots to shoots, resulting in higher nitrate levels in the shoots of treated cucumbers (Mora et al., 2010). This effect correlates with changes in the concentrations of cytokinins (CK) and polyamines (PA), indicating a link between the distribution of active CK, mineral nutrients, and the photosynthetic metabolism. This is further supported by the role of CK-responsive transcription factors in controlling the division of chloroplast components (Okazaki et al., 2009). Furthermore, HS treatment influences the activities of other enzymes involved in nitrogen metabolism, such as nitrate reductase and enzymes related to

ammonium assimilation. This leads to improved uptake and assimilation of nitrogen at the root level, enhancing the plant's overall nitrogen metabolism (Jannin et al., 2012; Mora et al., 2010; Tavares et al., 2017).

In addition to nitrogen and carbon metabolisms, sulfur metabolism seems to be impacted by HS treatment. An increased sulfate content, 76% and 137% in rapeseed shoots and roots, respectively, was observed and correlated with an upregulation of genes involved on sulfur uptake, assimilation and transport (Jannin et al., 2012). Similarly, an increase of sulfate content has been observed in maize roots seedlings (Eyheraguibel et al., 2008).

Finally, HS treatment affects the uptake and distribution of various nutrients beyond carbon, nitrogen, and sulfur. For instance, it enhances the affinity of root phosphate transporters, leading to increased phosphate uptake (Jindo et al., 2016) and can influences the translocation of calcium, magnesium, and potassium from roots to shoots, resulting in elevated concentrations of these essential nutrients in the leaves (Arango et al., 2003; Mora et al., 2010; Rose et al., 2014). Despite the numerous studies explaining the effect of HA and FA by the activation of hormonal pathways, this phenomenon is probably not the only involved. Indeed, Mora et al. (2012) highlight that the effect on the whole root system is independent on the phytohormones IAA, NO and ET, suggesting that other molecule signals should be working in coordination to or independently of those HS-affected phytoregulators.

2.3. Direct effects on plant metabolism - ROS pathway

In addition to activation of the hormonal pathway, HS also impact directly the plant through the activation their ROS pathways. This activation is based on a physical process, HS, which partially accumulate at the root surface, could trigger a colloidal mild stress and a reduction of *Lpr* by clogging pores (Berbara and Garcia, 2014). HS accumulation may act as a mild stress, priming the plant by activating the antioxidant system. This mechanism could explain how diverse structural molecules could induce similar mechanisms in plants (Olaetxea et al., 2015). This hypothesis is supported by the experiment of Asli and Neumann (2010) where after 70 min of HA (1 $g.L^{-1}$) accumulation at root cell-walls, a reduction of 44% in hydraulic conductivity of maize roots was observed due to a reduction in estimated cell wall pore diameters. In addition, the coloration of roots by the biostimulant suggests an accumulation on or in the cell wall pores.

This interaction is detected by the ROS generation mechanisms and led to an increase of ROS and antioxidant enzymes activities in roots and leaves (García et al., 2012; Schiavon et al., 2010). Activated antiox-

idative enzymes impacted expression of second metabolisms involved in stress alleviation. Increase in the expression of phenylalanine ammonia-lyase/ tyrosine ammonia-lyase (*PAL/TAL*) was observed by Schiavon et al. (2010), inducing an accumulation of phenol in leaves which protects them. The activation of both ROS and antioxidative enzymes allowed the induction of a signal without degrading plant cell with lipid peroxidation (García et al., 2012).

In parallel, increase of H_2O_2 level in root differentiation and of O_2^- in root elongation induced cell loosening and increased membrane permeability and hyperpolarization (Berbara and Garcia, 2014). This hyperpolarization generates a pH signal that modulates Ca^{2+} signaling by stimulating Ca^{2+} channels and increasing Ca^{2+} influx (Demidchik et al., 2007). The creation of a Ca^{2+} gradient in the apical root region stimulated secondary root growth increasing root hairs growth (Šamaj et al., 2004). Moreover, calcium ions are recognized to represent important second messengers in many signaling pathways. Increased cytosolic calcium concentration activates a variety of cellular responses like calcium-dependent protein phosphorylation (CDPK) at transcriptional and biochemical levels. Ramos et al. (2015) observed in lateral root in rice seedlings an increased expression of two stress responsive CDPK isoforms (*OsCPK7* and *OsCPK17*) after HS treatment. They indicated HS as a molecular elicitor of H^+ and Ca^{2+} fluxes leading to a CDPK activation cascade. CDPKs are involved in environmental stress signaling and improve plant tolerance to abiotic stresses. For example, increased CDPK transcripts were observed in *Arabidopsis* exposed to cold, salt, and drought stress. Additionally, rice plants tolerant to salt and drought stress overexpressed *OsCPK7*(Saijo et al., 2000; Urao et al., 1994).

2.4. Crosstalk between hormonal and ROS pathways

The study of the effects and mode of action of HS reveal that beneficial action of HS is complex and involves non-linear, cross-interrelated, and dynamic processes linked to both hormonal networks and secondary messengers (ROS, Ca^{2+}) (García et al., 2016). Between the hormonal and the ROS pathways, some molecules such as ABA, calcium and ROS are involved in both and could maybe be crosstalk.

Research has shown that ABA plays a pivotal role in plant physiology by influencing both root hydraulic conductivity and shoot growth through activation of plasma and tonoplast aquaporins (Mora et al., 2010; Olaetxea et al., 2015). In addition, its regulation is directly involves in crosstalks with IAA- and ethylene-dependent pathways (Mora et al., 2014). Furthermore, ABA interacts with ROS and calcium signaling by contributing to the production of H_2O_2 and the stimulation of Ca^{2+} channels, crucial for stomatal operations (Schmidt et al., 2014;

Suzuki et al., 2014). For example, under osmotic stress, enhancement of auxin transport by ABA leads to increased H^+-ATPase activity and root elongation, showing how ABA mediates hormonal and stress responses through ROS and Ca^{2+} pathways (Xu et al., 2013).

ROS signalling plays also a vital role in the mechanism of action of HS. Indeed, ROS production following hyperpolarization of membranes triggers the activation of Ca^{2+} channels (Demidchik et al., 2007). This calcium influx can inhibit H^+-ATPase activity, induced by auxin pathway. This affects cell expansion and differentiation in the root zone demonstrating how calcium efflux can transiently expand cells before inhibiting this expansion through calcium signaling (Ramos et al., 2015). Moreover, ROS are involved in regulating the transcripts of auxin receptors and transcriptional repressors of auxin, illustrating how ROS pathways are integrally connected with hormonal signaling to regulate plant growth and response mechanisms (Blomster et al., 2011).

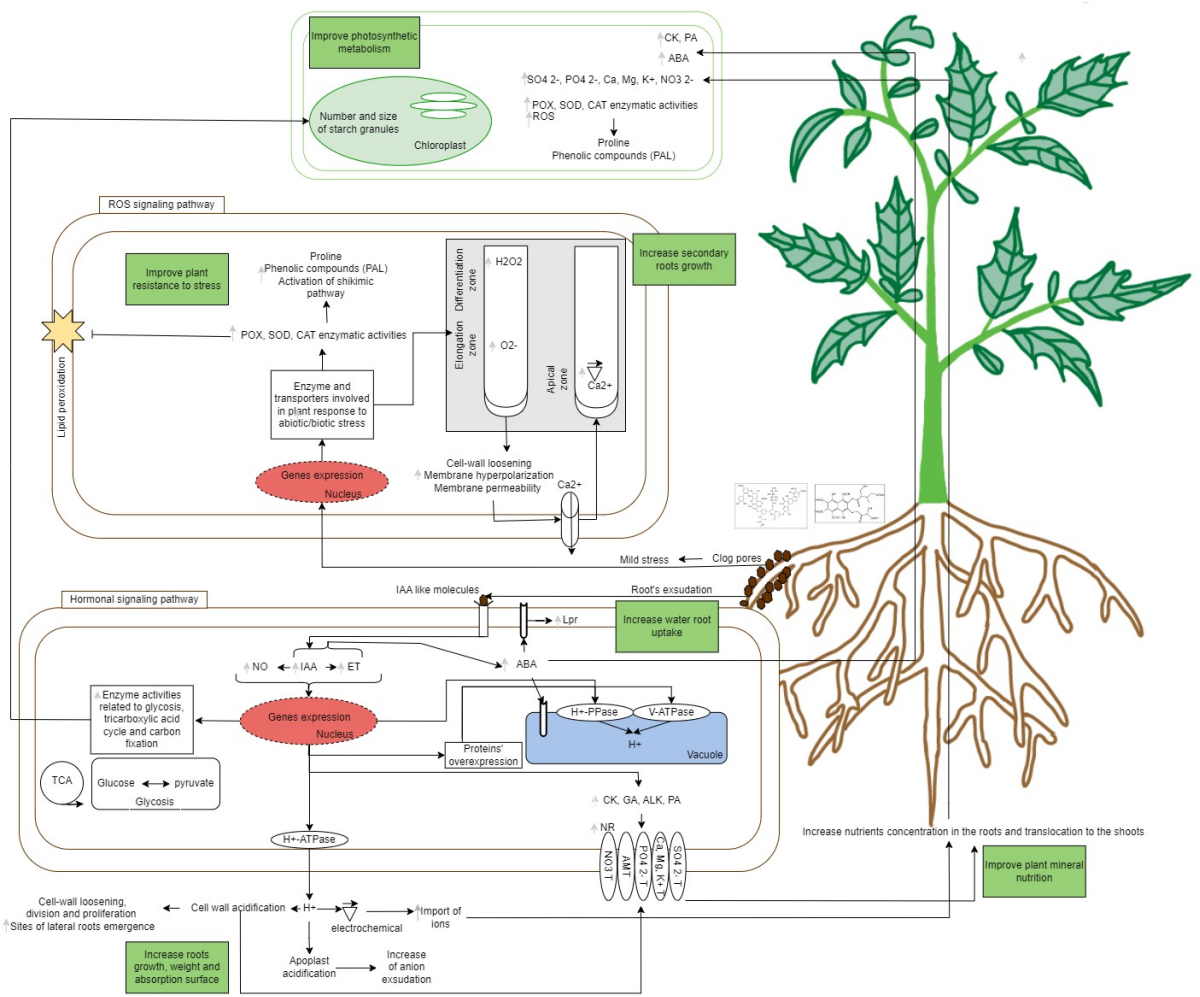

Figure IV-4: Schematic representation of directs effects of humic substances used as biostimulant on plants.
CK : cytokinins, GA : gibberellic acids, ALK : Alkaloides, PA : polyamines; NR : nitrate reductase; NO : nitric oxyde; IAA : indole-3-acetic acid, ET : ethylene; Lpr : root hydraulic conductivity; POX : peroxydase, SOD : superoxyde dismutase; CAT : catalase; ABA : abscisic acid

3. Complementation effects of bacteria and humic

Although HS are effective as stand-alone biostimulants, numerous studies have shown that their positive impact on plant growth and stress tolerance is enhanced when used in association with beneficial bacteria (Table IV-2) (Canellas et al., 2013, 2015; dos Santos et al., 2021; Olivares et al., 2015). For example, Galambos et al. (2020) reported that the combined use of HS with *Paraburkholderia phytofirmans* and *Pantoea agglomerans* significantly promoted tomato growth, suggesting a synergistic effect. This finding aligns with research by de Melo et al. (2018) and Olivares et al. (2015), which demonstrated improved plant vitality and stress tolerance when HS were used alongside microbial inoculants. In maize, Canellas et al. (2013) found that applying HS with *Herbaspirillum seropedicae* activated key metabolic pathways, enhancing the plant's response to environmental challenges. Similarly, Baldotto et al. (2010) observed that such co-inoculation strategies led to significant improvements in nutrient uptake and overall plant health. Ekin (2019) showed that *Bacillus* species combined with HS under different watering conditions significantly increased safflower yield, echoing the positive outcomes reported by Young et al. (2006) in lettuce and Feoktistova et al. (2023) in wheat under water stress conditions.

The synergistic effects of HS and PGPB on plant-bacterial interactions involve several key mechanisms. HS modulate plant-bacteria interactions primarily by altering plant morphology and physiology, thereby enhancing bacterial colonization. dos Santos et al. (2021) highlighted the role of HS in facilitating bacterial colonization, which in turn contributes to better plant growth and stress management. Initially, HS promote the production of lateral roots and root hairs, increasing the root surface area which, in turn, facilitates bacterial attachment and entry through openings created during root development (Nardi et al., 2009; Olivares et al., 2015). Additionally, the adherence of HS to plant surfaces, including roots and leaves, creates a more heterogeneous environment that aids bacterial anchoring (Olivares et al., 2017). Furthermore, HS serve protective and transport roles, incorporating organisms such as bacteria within their hydrophobic domains (Piccolo, 2002). Finally, HS may induce physiological changes in plants, such as the increased exudation of organic acids from maize roots, which subsequently augments the carbon source available for bacteria, enhancing the rhizosphere population, chemotaxis and thus endophytic communities (Canellas et al., 2008; Puglisi et al., 2008). This phenomenon has been observed by Canellas et al. (2013) where the co-inoculation of endophytic diazotrophic bacteria *Herbaspirillum seropedicae* with HA and FA increased the number of viable bacteria cells within the plant.

Table IV-2: Effects of co-inoculation of humic substances and bacteria on plant growth

Plant species	Humic Substances	Bacteria	Effects on Plants	References
Maize	HA from organic compounds obtained from bovine manure (23 $mmol.L^{-1}$)	*Rhizobium cellulosilyticum* (131x10^3 $CFU.mL^{-1}$)	Increase growth of plantlets and *R. cellulosilyticum* population	(de Melo et al., 2018)
Tomato	Soluble HS from vermicomposted cattle manure (20 mg $C.L^{-1}$)	*Herbaspirillum seropedicae* HRC54 (5 × 10^8 $cells.mL^{-1}$)	Increase of fruit biomass, enhanced nitrate uptake and nitrate reductase activity; stimulation of the secondary metabolic phenylalanine ammonia lyase pathway	(Olivares et al., 2015)
Lettuce	HA from a peat soil (0.01% and 0.001% w/v)	*Bacillus subtilis* CC-pg104 (4.10^8 $CFU.ml^{-1}$)	Plant growth promotion	Young et al., 2006
Wheat	Humate from brown coal (0.1% aqueous solution)	*Pseudomonas plecoglossicida* 2,4-D (10^8 CFU mL^{-1})	Plant growth promotion under water deficit with increased root growth, chlorophyll and flavonoid concentrations	(Feoktistova et al., 2023)

TableIV-2 (continued)

Plant species	Humic Substances	Bacteria	Effects on Plants	References
Maize	HS isolated from vermicompost (10, 20, or 30 mg.C L^{-1})	*Herbaspirillum seropedicae* (10^9 cells.mL^{-1})	Enhancement of plasma membrane H+-ATPase activity, alteration of sugar and N metabolism, and greater net photosynthesis; Increase of the number of viable bacterial cells	(Canellas et al., 2013)
Pineapple	HA isolated from vermicompost (15 mmol.L^{-1} of C)	*Burkholderia* sp. UENF 114111 and *Burkholderia silvatlantica* UENF 117111 (10^8 cells mL^{-1})	Improved shoot and roots growth with an increase concentration of N, P, K in leaves	(Baldotto et al., 2010)
Potato	HA from leonardite (200, 400, and 600 kg ha^{-1})	*Bacillus megatorium* and *Bacillus subtilis* (10^9 CFU.mL^{-1})	Increased potato growth, tuber yields, and quality	Ekin, 2019
Soybean	K-humate brown coal (50 mg.L^{-1})	*Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA 5080 strains (10^9 CFU.mL^{-1})	Increased nodulation, biomass and N content in the shoots	(dos Santos et al., 2021)

TableIV-2 (continued)

Plant species	**Humic Substances**	**Bacteria**	**Effects on Plants**	**References**
Tomato	HA (50 mg.L^{-1})	*Paraburkholderia phytofirmans* PsJN, *Pantoea agglomerans* D7G, *Enterobacter* sp. 32A (1×10^7 CFU.ml^{-1})	Promoted tomato shoot growth, without impacting colonization by bacterial strains	(Galambos et al., 2020)
Tomato	HA (500 mg. L^{-1})	*Bacillus cereus* SA1(10^9 CFU.mL^{-1})	Increased shoot and root length, higher P, K, Fe concentration, increased fresh and dry weight and chlorophyll fluorescence. Alteration in salicylic acid	(Khan et al., 2020)
Safflower	HA	*B. megaterium* M3 and *B. subtilis* OSU142 (10^9 CFU.mL^{-1})	Growth promotion	(Ekin, 2020)
Maize	HA isolated from vermicompost	*Herbaspirillum seropedicae*	Increased extracellular H+ flux, changed electrochemical potential and overexpression of aquaporins	(de Azevedo et al., 2019)

However, the complementary effect of HS and bacteria is not always explained by easier bacterial colonization. In the study of Galambos et al. (2020), PGPB-coated tomato seeds grown in hydroponics had a greater shoot length when HS and PGPR were co-applied even if the bacterial colonization was unchanged. In this case, HA application affected growth promotion effects and transcriptional responses activated by bacterial endophytes in tomato plants (Galambos et al., 2020). Similary, in the study of Aguiar et al. (2016), the beneficial effects of HA and PGPB on sugarcane during drought stress did not derive from enhanced plant colonization by bacteria but from distinct physiological responses. Sugarcane treated with PGPB showed a preservation of leaf water potential likely due to a "water preservation" strategy involving stomatal closure. This response was facilitated by decreased ethylene production via ACC-deaminase activity and stable ABA levels. In contrast, plants treated with HA did not exhibit reduced water potential, suggesting that their stomata remained open. It is hypothesized that these plants coped with stress by boosting antioxidant enzyme activities, with increased levels of enzymes like SOD, CAT, and APX observed. This protective effect might be linked to an auxin-like action of HA, which keeps stomata open. However, when plants were treated with both PGPB and HA, they exhibited higher water retention and maintained efficient photosystem function during stress, suggesting a novel response to rehydration and stress conditions (Table IV-3) (Aguiar et al., 2016).

Table IV-3: Effects of HA, PGPB or both treatments on sugarcane growth under growth stress (Aguiar et al., 2016)

Parameters	**HS**	**PGPB**	**HS + PGPB**
Shoot dry weight	+	+	++
Root dry weight	++	+	+++
% N	+	++	+
Relative water content	+	++	++
Water potential in leaves	0	-	- - -
Stomatal conductance	++	-	+
Photosynthetic rate	++	-	+
Antioxidant enzymes	+++	-	-

To conclude, the beneficial effects of HS and PGPB are likely due to multiple factors: (1) increased bacterial colonization and penetration, (2) activation of plant metabolic pathways that complement those induce by bacteria and HS individually, (3) direct effect of HS on bacterial metabolism. Since HS are known to influence co-inoculated bacteria, it

stands to reason that they would similarly impact the communities of endophytic bacteria within plants.

4. Interaction between HS and bacterial endophytome in hydroponics

In order to study the direct effect of HS on the bacterial endophytome, we concentrated on experiments carried out in hydroponic systems in this section. As explained in the section 2.2 and 2.3, HS play an essential role in modulating the hormonal and redox metabolism of plants, thereby improving their growth and enhancing their tolerance to abiotic stresses. Although relatively few studies have specifically examined the influence of HS on the endophytome, existing research indicates a significant modulating effect (Table IV-4). For instance, when soybean plants were treated with HS from vermicompost and millicompost under water-restricted conditions, plant growth and nodulation were enhanced in parallel with an increased root bacterial richness, particularly under non-stress conditions (Matuszak-Slamani et al., 2022). Similarly, in hydroponic systems, rice and cucumber plants treated with HS exhibited substantial shifts in their microbial communities (da Silva et al., 2021b; De Hita et al., 2018).

In the experiment of da Silva et al. (2021b), rice plants growing in hydroponics were treated every 72h with HA applied directly in the hydroponic solution. After 240h of treatment, plants were harvested and metabarcoding analysis revealed that root bacterial community structure and composition were affected upon HA application. Indeed, Bacteroidetes (especially genera *Mucilaginibacter* and *Chitinophaga*), *Pseudomonas* and Acidobacteria population were enriched while Proteobacteria and Actinobacteria communities were reduced. Slight variations also appeared in shoot communities with an increase of abundance of Proteobacteria and a decrease of Bacteroidetes and Actinobacteria (Figure IV-5). A network analysis revealed that HA-treated plant roots exhibited a more complex bacterial network with increased nodes and connections, especially positive interactions, compared to controls. This suggests enhanced community connectivity possibly due to HA's influence on plant metabolism or direct stimulation of the microbiota (da Silva et al., 2021b). In parallel, cucumber seedlings grown in hydroponics and treated with HA applied at the root level shown an increase of Firmicutes presence in their endophytic communities and the absence of minor groups such as Pavachaeota, Pacearchaeota, Fusobacteria or Gemmatimonadetes (De Hita et al., 2018)

Table IV-4: Effects of HS on the bacterial endophytome of hydroponically-grown plants

Plant-associated microbiota	Stress	Simplified assay description	Effects	References
Endophytes	No	Cucumber seedlings grown in hydroponics and treated with humic acid (extracted from leonardite) applied at the root level.	Increase in Firmicutes presence in treated plant and the absence (or barely presence) of minor groups such as *Fusobacteria* were observed. At the class taxonomic level, major changes appeared in some agriculturally important groups as Bacilli, Bacteroidia, Clostridia, and Sphingobacteriia. No difference was observed in fungal communities between treated and control plants.	(De Hita et al., 2018)
Endophytes	No	Rice plants growing in hydroponics treated with HA in the hydroponic solution.	Root bacterial community structure and composition were affected upon HA application. Bacteroidetes (especially genera *Mucilaginibacter* and *Chitinophaga*), *Pseudomonas* and Acidobacteria population were enriched while Proteobacteria and Actinobacteria communities were reduced. Slight variations in shoot communities with an increase of abundance of Proteobacteria and a decrease of Bacteroidetes and Actinobacteria	(da Silva et al., 2021b)

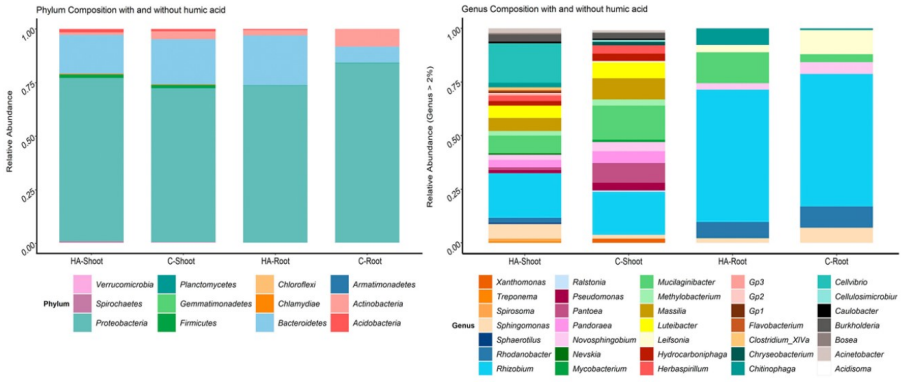

Figure IV-5: Relative abundance of bacterial taxa in roots and shoots of plants treated with humic acids classified at phylum and genus levels.

In the case of genus, only those taxa with a relative abundance above 2% are displayed. C-shoot, C-root, HA-shoot, and HA-shoot stand for shoot and roots of the control and humic acid-treated plants, respectively (da Silva et al., 2021b)

Despite these promising observations, the precise mechanisms by which HS influence these microbiota and their ultimate impact on plant health remain poorly understood. There is a clear need for more controlled experimental studies that can dissect the individual contributions of HS to soil health, endophytic interactions, and plant physiology. Such studies would help clarify the direct versus indirect effects of HS on plant growth and microbial dynamics, providing deeper insights into their potential as biostimulants in agriculture.

OBJECTIVES

Despite the important roles of seed-transmitted endophytic bacteria in plant physiology, studies investigating the effects of HS on these bacterial communities under stress conditions remain scarce. This gaps highlight the need for more comprehensive studies using advanced metagenomic tools that can elucidate the complex relationships within plant holobionts, potentially leading to more effective biostimulation strategies.

This thesis aims to fill these gaps by examining the impact of HS on vertically-transmitted bacteria of tomato plants under stress conditions, addressing the following questions:

1. What is the composition of the bacterial core microbiota vertically-transmitted in tomato ?

2. What are the dynamics of vertically-transmitted endophytic bacterial colonization in tomato plants and how they improve tomato osmotic stress tolerance ?

3. What are the effects of HS on tomato plant growth, on plant osmotic stress tolerance, and on root vertically-transmitted endophytic bacteria?

To answer these questions, we will first develop a sterile hydroponic culture system to study seed-transmitted endophytic bacteria. Metabarcoding and culture-dependent techniques will provide a comprehensive view of the composition of seed-transmitted endophytic bacteria. Second, we will evaluate the ability of seed-transmitted bacteria to enhance tomato drought stress tolerance both *in vitro* and *in planta*, using mCherry-tagged bacterial strains to visually track colonization patterns across plant tissues. Finally, we will assess the impacts of HS on tomato growth and osmotic stress tolerance through morphological and physiological analysis. In parallel, impact of HS on root endophytic bacterial communities will also be investigated through high-throughput sequencing analyses.

RESULTS

CHAPTER V. Seed-borne endophytic bacteria of tomato – dynamic colonization and implication in plant health

Salomé Lengrand[1], Benjamin Dubois[2], François Renoz[1], Olivier Lebbe[1] Frederic Debode[2] and Anne Legrève[1*]

[1] Université catholique de Louvain (UCLouvain), Earth and Life Institute, Louvain-la-Neuve, Belgium,
[2] Walloon Agricultural Research Centre (CRA–W), Gembloux, Belgium

[*]Correspondence: Anne Legrève (anne.legreve@uclouvain)

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1. Abstract

Seed-borne endophytic bacteria are essential for seed germination, plant growth and stress tolerance. However, their functional diversity remains elusive due to difficulties in maintaining sterile conditions and amplifying bacterial community without amplifying plant genome. Using tomato (*Solanum lycopersicum* L.) as a model, we designed a novel culture system to study this bacterial community from seed to seedling, investigating their colonization and impact on plant health. Our workflow combined metabarcoding and culture-based approaches to capture the most accurate picture of the tomato seed-associated bacteria. Advanced metabarcoding protocol, involving sequencing of the 16S-ITS-23S *rrn* operon and the use of a newly designed tomato-specific PNA clamp var. Moneymaker, revealed a core microbiota consistently transmitted from seeds to shoots and roots. Key bacteria included *Ralstonia pickettii*, *Sphingomonas aerolata*, *Bradyrhizobium* sp., *Paucibacter aquatile*, *Microbacterium* sp. and *Stenotrophomonas* sp. Culture-dependent approaches isolated endophytic strains, like *Rummeliibacillus stabekisii*, *Peribacillus frigoritolerans* and *Pseudomonas protegens*, which increase plant osmotic stress resistance. Using quantitative PCR, confocal microscopy and colony counting, we demonstrated that *P. protegens* tagged with mCherry colonizes root, shoot, and leaf tissues. Overall, our study supports the hypothesis that a rich and stable core of endophytic bacteria, capable of enhancing drought resistance, is transferred from seeds to plant organs. Our study provides the most detailed investigation to date of the diversity of the seed-borne microbial community of tomato and offers new approaches to better assess the diversity of plant-associated microorganisms, a key step towards leveraging it to improve crop resistance and productivity.

2. Introduction

Plants are increasingly being recognized not as individual entities, but as complex ecosystems, known as 'plant holobionts'. These holobionts consist of the plant itself and its associated microbiota, which is highly diversified and inhabits various plant microhabitats such as the rhizosphere, phyllosphere, and endosphere (Hassani et al., 2018; Lemanceau et al., 2017; Müller et al., 2016). The understanding of endophytes has significantly evolved from being seen as harmless plant inhabitants to a diverse community of microorganisms that inhabit plants either obligatorily or facultatively within plants, interacting dynamically within the hosts (Bary et al., 1866; Lengrand et al., 2024). Endophytes, which encompass bacteria, fungi, archaea, and viruses, form a complex network of interactions that contribute to plant adaptation and survival in diverse environments. Indeed, several microbial endophytes have biostimulant properties (Pati and Rathore, 2024). It is important to note, however, that not all endophytes are beneficial to the plant; some may have neutral or even detrimental effects on their host (Hardoim et al., 2015). Endophytes can be transmitted vertically, i.e., from parent to offspring via seeds, or horizontally, i.e., acquired from the environment, highlighting the flexibility of plant-microbe interactions (Hardoim et al., 2015; Truyens et al., 2015). While extensive research has illuminated the microbial dynamics within shoot and roots habitats, the seed endophyte microbiome remains less explored (Nelson, 2018; Sanchez-Lopez et al., 2018; Truyens et al., 2015).

Studying seed microbiota is crucial as it constitutes the primary microbial inoculum for plants, offering early advantages that enhance growth and resilience to stresses, impacting subsequent plant generations (Rosenblueth et al., 2012; Truyens et al., 2015; Verma, 2019). Some taxa, such as *Pantoea agglomerans*, *Pseudomonas viridiflava*, and *P. fluorescens*, are commonly found in the seeds of many plant species (Simonin et al., 2022). However, research on seed microbiota faces challenges such as differentiating true endophytes from contaminants and understanding the dynamics and specific roles of microbial communities. Additionally, inconsistencies in methodology for sample preparation and sequencing make it challenging to compare findings across studies.

Tomato (*S. lycopersicum*) is one of the most significant agricultural crops, with global production reaching 186.1 million metric tons in 2022 (FAOSTAT, 2024). Although it plays a crucial biological role in the plant, the seed microbiota of tomatoes remains largely unknown. While Bergna et al. (2018) used 16S V4 sequencing to compare seed and root endophytic communities, most studies focus solely on seed microbiota or rely on cultivation-based methods (Sanchez-Lopez et al., 2018; Shaik and Thomas, 2019; Sharma and Kumar, 2021; Thomas and Shaik, 2020).

Typical cultivable members of seed-transmitted endophytic bacteria include *Bacillus*, *Micrococcus*, *Paenibacillus*, *Pantoea*, and *Pseudomonas* (Sanchez-Lopez et al., 2018; Sharma and Kumar, 2021). A thorough understanding of the tomato seed microbiota and its functions is essential to unlocking its potential impact on plant health and productivity. Indeed, despite the crucial biological role of the tomato seed microbiota, many questions remain unanswered in this field. For instance, what is the composition of the core microbiota that is transmitted from the seed to all plant organs? It is also unclear whether all seed-borne endophytic bacteria can colonize various microhabitats such as roots and shoots, or if there is a distinct distribution within each microhabitat. Another important question is whether seed-borne endophytic bacteria transition to becoming epiphytes, and if the same genera are involved in epiphytic colonization in both shoots and roots. Furthermore, it remains to be determined whether certain isolated endophytic bacteria are able to recolonize the plant when coated on seeds or applied to roots, the pathways they use for colonization, and whether they can colonize all plant organs or are confined to specific microhabitats.

In this study, we set out to establish the most comprehensive microbiota profile of tomato seeds-transmitted endophytic bacteria in seed and growing plants (Figure V-1). We first developed a sterile culture system approach that enabled us to accurately characterize the seed-transmitted bacterial microbiota of *S. lycopersicum* var. Moneymaker (Figure V-1,1). During seed germination, seed-borne bacteria may either colonize the plant as endophytes or exit to become epiphytes or rhizosphere colonizers (Hardoim et al., 2012). To explore these dynamics, we concurrently examined bacterial communities using 16S-ITS-23S *rrn* operon sequencing in both seeds and plants, which were either subjected to surface disinfection or left untreated (Figure V-1,2). Additionally, we employed culture-dependent methods to investigate seed-transmitted endophytic bacteria in various seed and plant parts. Based on this methodology, we identified and assessed the plant growth-promoting traits of the isolates *in vitro*. Additionally, we evaluated the effects of selected isolates on plant vigor *in planta* (Figure V-1,3). Finally, we examined the colonization patterns of two previously isolated endophytic bacteria by coating seeds with mCherry mutants, tracking them in plants using quantitative polymerase chain reaction (qPCR), colony counting, and confocal analysis (Figure V-1,4).

3. Material and Methods

3.1. Plant material and sterile growth conditions

To obtain the most accurate seed-microbiota composition possible, we established a protocol for tomato cultivation in sterile conditions. Tomato seeds (*S. lycopersicum* var. Moneymaker) were surface sterilized by immersing in less than 5% sodium hypochlorite for 10 minutes under orbital shaking and rinsed three times with sterile distilled water for 20 minutes. Seeds were then germinated in a sterile box for ten days, seven days in the dark and three days under light, photoperiod (16:8) in a germination chamber at 22° C, and 70% relative humidity. At the two cotyledons stage, the seedlings were transferred into a custom-designed sterile hydroponic system which consisted of two Erlens (150 mL) filled with Hoagland's solution adapted for tomato plants (NH_4NO_3: 0.04 g.L^{-1}; $Ca(NO_3)_4H_2O$: 0.413 g.L^{-1}; KNO_3: 0.2035 g.L^{-1}; KH_2PO_4: 0.137 g.L^{-1}; $MgSO_4.7H_2O$: 0.123 g.L^{-1}; $MnSO_4.5H_2O$: 0.265 mg.L^{-1}; H_3BO_3: 0.7 mg.L^{-1}; $CuSO_4.5H_2O$: 0.075 mg.L^{-1}; $(NH_4)Mo_7O_{24}.4H_2O$: 0.004 mg.L^{-1}; $ZnSO_4.7H_2O$: 0.3 mg.L^{-1}; Fe EDDHA: 0.03 g.L^{-1}). This system was enclosed in an autoclavable culture bag with a 0.02 µm filter (Sun bag, transparent, B7026-100EA) to allow gas exchange, supported by a plastic frame and a sterile plant potholder, ensuring a controlled environment Figure V-1. Tomato plants were cultivated in a growth chamber under a 16h/8h (light/dark) photoperiod regime, 70% relative humidity, and a temperature of 24° C/22° C (day/night). The hydroponic solution was replenished under sterile conditions every week. After three weeks in the hydroponic system, the plants were harvested for DNA extraction and bacterial isolation on media.

The sterility of the system was checked at the harvest time by spreading hydroponic solution of a system without plant on Luria Broth Agar (LBA) media. Moreover, the possible contamination of the hydroponic solution with non-cultivable bacteria was evaluated using a molecular method. The DNA of hydroponic solution was extracted using a cetyltrimethylammonium bromide (CTAB) chloroform protocol followed by a PCR targeting the 16S rRNA gene (described in section 3.3).

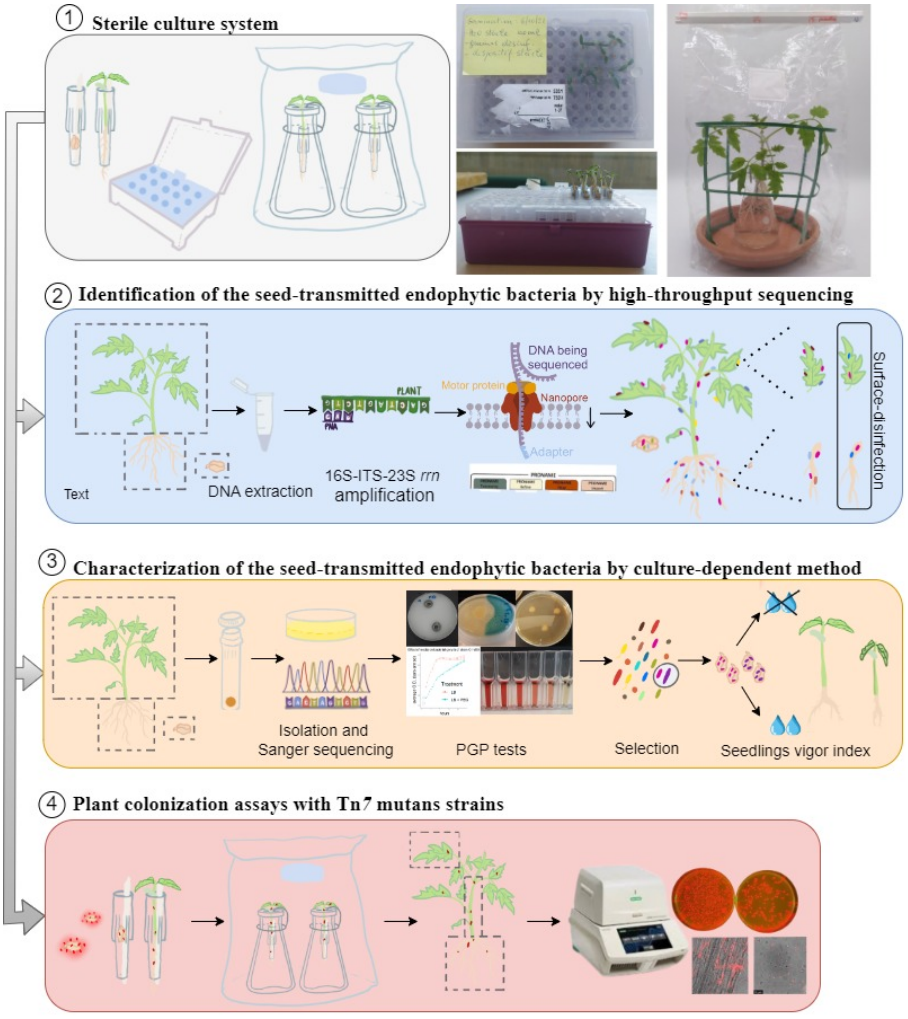

Figure V-1: Schematic of the experimental workflow.

(1) Germination and culture under sterile conditions (see section "Plant material and sterile growth conditions"), (2) DNA extraction and sequencing (see section "Identification of seed-transmitted endophytic bacteria by metabarcoding"), (3) Isolation and identification of seed-borne endophytic bacteria (see section "Identification and characterization of seed-transmitted endophytic bacteria by culture-dependent method"), (4) two previously isolates were transformed in mCherry mutants to evaluate their plant colonization pattern (see section "Plant colonization assays with Tn7 mutants strains").

3.2. Identification of seed-transmitted endophytic bacteria by metabarcoding

DNA extraction from seeds, shoots and roots

To identify seed-transmitted endophytic bacteria and then to map their distribution within plant organs and identify their colonization mode (i.e., endophytic versus epiphytic), endophytic communities of seeds, roots, shoots (which encompass all the aerial parts) of plant surface-disinfected or not were analyzed. Three aliquots of disinfected seeds were immersed separately into liquid nitrogen and ground in sterile conditions. Three plants were directly separated into shoots and roots and ground into liquid nitrogen and three others were surface-disinfected. Briefly, entire tomatoes were first placed in NAP buffer (124 mM Na_2HPO_4) and sonicated in an Ultrasonic Cleaning Baths (VWR®) for 1 min to dislodge surface bacteria. Subsequently, plants were then submerged in sodium hypochlorite solution (5.25%) for 6 min and rinsed with sterile water (Ruiz-Perez and Zambrano, 2017). The intact plants were dried on sterile paper under sterile conditions. The sterilization was checked by making an imprint of plants on LBA and plates were checked for two weeks (Fitzpatrick et al., 2018b).

Following this, shoots and roots were separated and ground into liquid nitrogen. Each powdered sample, comprising three pools of seeds, three surfaced-disinfected roots and shoots and three non-disinfected roots and aerial parts, underwent a DNA extraction using a modified CTAB-based method to facilitate plant tissues extraction. Briefly, NaCl (100 µL, 5M) was added before isoamyl alcohol: chloroform to reduce high polysaccharide concentration in the extractant (Fang et al., 1992). The DNA pellet was diluted in 20 µL of sterile nuclease-free water.

PCR amplification of ribosomal markers of endophytic bacteria with universal Peptide Nucleic Acid (PNA) clamps

Extracted DNA was amplified using the primers 16S-8F (5'-AGRGTTYGATYMTGGCTCAG-3') and 23S-2490R (5'-CGACATCGAGGTGCCAAAC-3') targeting the 16S-ITS-23S of the ribosomal operon (*rrn*) (˜4,500 bp) (Karst et al., 2021). Their non-selectivity was tested by comparing the reads amplified with three other pairs of primers commonly used (Table S-1). The PCR mix (25 µL total volume) contained 2×GoTaq Long PCR Master Mix (Promega), 0.4 µM of 16S-8F primer, 0.4 µM of 23S-2490R primer, 2 µM of mPNA (to block mitochondrial amplification), 2 µM of pPNA (to block plastid amplification), and 20 ng of DNA (Lundberg et al., 2013). The PCR thermal profile consisted

of an initial denaturation of 2 min at 95° C followed by 30 cycles of 20 s at 95° C, 60 s at 74° C, 30 s at 55° C, 5 min at 72° C and a final extension of 10 min at 72° C. For each sample, PCR was performed in three replicates and pooled before purification to assure homogeneity. The amplicons of the 16S-ITS-23S *rrn* operon were cleaned up with AMPure XP beads (Beckman Coulter) using a 0.5X beads-to-sample ratio.

Illumina sequencing and design of a specific PNA clamp for *S. lycopersicum* var. Moneymaker

To assess the proportion of plants and bacterial sequences in amplified DNA, an Illumina sequencing of the V1-V3 region of the 16S rRNA gene (for: 5'-AGAGTTTGATCATGGCTCAG-3' and rev: 5'-GTATTACCGCGGCTGCTG-3') was performed by Eurofins Genomics directly on the *rrn* amplicons of three plant samples (Leser et al., 2002; Weisburg et al., 1991). This is a crucial step because amplification of the *rrn* operon, even with the use of universal PNA clamps, can also inadvertently amplify the genome of tomato. This step therefore makes it possible to increase the proportion of reads attributed to the bacterial microbiota compared with those from the host plant, thereby increasing the accuracy of the microbial community mapping. After Illumina sequencing, demultiplexed sequence files were imported into QIIME2 (2023.2) prior to visualization (Bolyen et al., 2019). Data were quality-filtered, dereplicated, and error-corrected using 'q2-dada2'(Callahan et al., 2016). Representative sequences were used for taxonomy classification using qiime2 feature-classifier classify-sklearn using the Silva v138 reference database (Quast et al., 2012). Then, the 'qiime taxa filter-table' command was used to remove amplicon sequence variants (ASV) from the table that were identified as chloroplast or mitochondria sequences. This allowed evaluating the number of reads associated with the host plant *S. lycopersicum* and with bacteria.

To identify DNA sections that are different between tomato and bacteria sequences, the plant-derived amplicons were aligned with 60 sequences of V1-V3 region of the 16S rRNA of bacteria belonging to diverse phyla, genera, and species often found in endophytic bacterial communities (Table S-2). One sequence of PNA was selected based on the criteria required for PNA functionalities (`https://www.pnabio.com/`). *In silico* verification was performed using Basic Local Alignment Search Tool (BLAST) against the nr National Center for Biotechnology Information (NCBI) database to verify that the PNA would not block the amplification of any bacteria. Moreover, DNA extract from three plants were amplified and sequenced with and without the use of the new PNA. Amplification and sequencing were done using the same protocol as described in previous sections.

Library preparation and Oxford Nanopore sequencing

Endophytic communities of seeds, roots, and shoots were amplified using three PNA clamps (mPNA, pPNA, and the new PNA-chr11). The PCR mixture was modified by adding 2 µM of PNA-chr11, and the PCR thermal profile was identical to that described above. Each sample was amplified in triplicate and pooled before the clean-up step with AMPure XP beads (Beckman Coulter) using a 0.5X beads-to-sample ratio. The amplicon concentration and quality were evaluated using a QuantusTM Fluorometer (Promega) and a Nanodrop spectrophotometer (ThermoFisher Scientific), respectively. PCR products were visualized on a 0.8% agarose gel.

Libraries were prepared using the Ligation Sequencing Kit V14 (SQK-LSK114) (Oxford Nanopore Technologies) according to the manufacturer's instructions. Briefly, amplicons were processed for end repair using the NEBNextUltra II End Repair/dA-tailing Module (New England Biolabs) and sequencing adapters were attached. Libraries were sequenced during 24h using Flongle Flow Cells (R10.4.1) and a MinION device (Oxford Nanopore Technologies).

Data processing and analysis of diversity

The raw data were processed using PRONAME (Dubois et al., submitted to Frontiers in Bioinformatics). Initially, the data were imported into PRONAME, where adapters and primers were trimmed. Subsequently, a graph illustrating the length vs. quality distribution of simplex and duplex reads was generated. The data were then filtered to retain only duplex reads that were between 3500 and 5000 base pairs in length and had a minimum quality score of 15. PRONAME further clustered reads together by setting a 90% identity threshold and polished the centroid sequences with Medaka, using 300 reads subsampled from each cluster. Finally, chimera sequences were filtered out and the representative sequences were subjected to a BLAST analysis against the rEGEN-B database, a curated and extensive reference database dedicated to bacterial *rrn* operon sequences, also included in the PRONAME package. The analysis of data composition was carried out using the physeq package (McMurdie and Holmes, 2013) in RStudio, and the results were visualized using the ggplot2 package (Wickham, 2014). For alpha diversity, the vegan package was utilized to calculate the Observed features, Shannon, Simpson, and InvSimpson indices, and the results were statistically evaluated using the Wilcoxon test with Benjamini-Hochberg (BH) adjustment for multiple comparisons. Beta diversity was examined using Principal Coordinates Analysis (PCoA) with the ape package (Paradis, 2024) and further analyzed through the analysis of similarity (ANOSIM)

test employing the vegan package (Oksanen and Simpson, 2009). Additionally, Venn diagrams were generated using the VennDiagram package (Chen and Boutros, 2011).

3.3. Identification and characterization of seed-transmitted endophytic bacteria by culture-dependent method

A pool of four seeds was weighed and cut with a sterile blade. The tomato plants were surface disinfected as described in section "DNA extraction from seeds, shoots and roots" prior to isolation of the endophytic bacteria. Six plants per treatment were separated into shoots (comprising all plant parts) and roots after the surface disinfection. Seeds, shoots, and roots were placed individually in 2mL tubes with phosphate-buffered saline (PBS) solution (NaCl: 8g.L^{-1}; KCl: 0.2g.L^{-1}; Na_2HPO_4.($12H_2O$): 2.9 g.L^{-1}; KH_2PO_4: 0.2g.L^{-1}) (European and Organisation, 2018). The tubes were homogenized twice at room temperature in a Fast prep (30s) and dilutions (10^{-1}, 10^{-2}, 10^{-3}) were spread on four different nutrient-rich levels of media: nutrient agar (NA), LBA, V8, and Buffered Charcoal Yeast Extract (BCYE) media (Wells et al., 1981) supplemented with an antifungal (cycloheximide; 50 mg.L^{-1}) and incubated at 28° C for two weeks. Given that the tomatoes were grown in a sterile system, any bacteria present in the hydroponic solution must have originated from the seeds. Therefore, the hydroponic solution was sterilely pipetted at harvest, and 100 µL were spread on the same media (NA, LBA, V8, and BCYE).

For molecular identification, single colonies of each isolate were mixed in water and heated for 5 minutes at 100°C before ice shocking for bacterial DNA extraction. The 16S rRNA gene was PCR-amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR conditions were as follows: an initial denaturation for 5 min at 94° C; 30 cycles (1 min at 94° C, 30s at 61° C, and 1 min 30 at 72° C); 10 min at 72° C and hold at 10° C. PCR products were checked on a 1.2% agarose gel and purified samples were sent to Microsynth (Balgach, Switzerland) for Sanger sequencing. All the sequences obtained were analyzed for homology searches using the BLAST algorithm against the nucleotide sequences present in the GenBank (NCBI) database. A phylogenetic tree was constructed using the Neighbor-joining method (Saitou and Nei, 1987) in Geneious (2023.0). Pre-cultures of the isolated bacteria were mixed with glycerol and stored at -80° C to form a collection reflecting the cultivable component of the tomato seed microbiota.

Characterization of cultivable bacterial endophytes

The initial screening of isolated bacteria for drought stress was conducted using the method described by Kumari et al. (2019). Briefly, bacterial precultures were incubated overnight at 28° C with shaking at 180 rpm. Subsequently, a 0.5 mL aliquot of each preculture was added to 4.5 mL of LB broth, with or without 10% w/v polyethylene glycol (PEG) and incubated under shaking at 28° C for five days. The viability of bacterial growth was monitored by measuring the optical density at 600 nm (O.D. 600). Strains that exhibited a growth ratio in LB versus LB+PEG of equal to or greater than 1, and those between 1 and 0.5, were selected for further analysis.

For these selected isolates, a more detailed assessment of drought stress tolerance was performed by comparing their growth curves in LB and LB supplemented with PEG. Cultures were initially carried out with LB, then centrifuged to discard the supernatant. The bacterial cell pellets were resuspended in fresh LB or LB+PEG to an O.D. 600 of 0.01. Growth was monitored at 28° C during 24 hours by measuring O.D. 600 hourly, with controls set up using non-inoculated LB and LB+PEG. This test was conducted twice, in triplicate, using 96-well microplates with 200 µL per well on a Multiscan FC (©Thermoscientific). Growth inhibition under osmotic stress was quantified based on the area under the growth curves, with inhibition rates categorized as follows: less than 25% inhibition indicated high tolerance, 25% to 50% indicated moderate tolerance, and greater than 50% indicated low tolerance.

Bacterial isolates were tested for indole-3-acetic acid (IAA) production using a colorimetric technique performed with Van Urk Salkowski reagent according to the Salkowski's method (Ehmann, 1977) by adjusting O.D. 600 of bacterial suspension at 0.1. Calibration curve was done with pure IAA ranging between 0 and 100 $\mu g.mL^{-1}$ diluted in LB (Gang et al., 2020). Uninoculated LB served as control. The experiment was repeated three times.

Exopolysaccharides (EPS) production was estimated as described by Paulo et al. (2012) using solid medium-containing discs of filter paper (5 mm diameter) that were saturated with active cultures (5 µL of culture adjusted to O.D. 600 of 0.1). EPS production was evidenced after seven days by the presence of mucoid colonies on the discs, which was confirmed by the formation of a precipitate when a part of this colony was mixed with absolute alcohol.

The ability of bacteria to solubilize precipitated phosphate was tested by inoculating 10 µL of an overnight bacterial culture in an artificial well on the Pikovskaya medium containing calcium phosphate. The formation of a clear halo around the well after incubation at 30° C for

7 days indicates a positive result (Thomloudi et al., 2021).

Siderophore production was evaluated by the Chrome Azurol S (CAS) agar assay (Schwyn and Neilands, 1987). A 10 µL volume of overnight culture was inoculated in the artificial well on CAS agar plate (Louden et al., 2011). Released siderophores were detected by the formation of orange halos on CAS agar plates after incubation for 7 days at 30° C.

Evaluation of clonal bacterial isolated with Random Amplified Polymorphic DNA analysis (RAPD)

RAPD of culturable bacteria was performed to check for clonal bacterial isolates using RAPD272 (5'-AGCGGGCCAA-3') (Hematzadeh and Haghkhah, 2021). The PCR conditions were as follows: an initial denaturation for 2 min at 94° C; 35 cycles (30s at 94° C, 30s at 35° C, and 2 min at 72° C) and a final extension of 10 min at 72° C. The profiles of strains with the same identification on NCBI Blast were compared, and the strains with the same identification, RAPD profile, and phenotype were considered as clones.

The identification of selected strains for *in planta* tests was refined by making a Multi-Locus Sequence Analysis (MLSA) targeting, in addition to the 16S rRNA, the gene *gyrB* for bacterial strains belonging to Firmicutes and genes *rpoB*, *rpoD*, and *gyrB* for *Pseudomonas* strains. PCR amplification conditions were as follows: initial denaturation at 94° C for 5 min, 30 cycles of denaturation at 94° C for 30 sec, annealing for 30 sec, elongation at 72° C for 60 sec, and a final extension step at 72° C for 5 min. The characteristics of primers and their annealing temperatures are detailed in Table S-3. Sequences were concatenated and queried against the RefSeq Representative Genomes database at the NCBI.

Assessment of bacterial coating effects on seed vigor index

For the nine selected bacterial strains based on Plant Growth Promoting (PGP) tests, a single colony was grown overnight in LB at 28° C with shaking at 180 rpm. The bacterial cultures were then centrifuged (5000 rpm, 10 min), the pellet was washed three times with PBS, and the O.D. 600 was adjusted to a concentration of 10^8 CFU/mL. Tomato seeds were disinfected according to previously described methods before incubation in the bacterial suspensions for 2 hours. Control seeds were similarly incubated in PBS alone. After coating, the seeds were placed on Whatman paper in Petri dishes, which were moistened with either sterile water or a 10% w/v PEG6000 solution to induce an osmotic stress of -0.2 MPa.

Each treatment, whether non-stress or osmotic stress conditions, was performed in triplicate with 12 seeds per Petri dish. The dishes were incubated in the dark at 22° C for seven days, then exposed to light for three days. After 10 days, the seedling vigor index was calculated using the formula described by Bhatt et al. (2015):

$$\text{Seed vigour index} = \%\text{germination} \times (\text{SRL} + \text{SL})$$

Where SRL = seedling root length (cm); SL = shoot length (cm)

3.4. Plant colonization assays with Tn*7* mutants strains

The colonization of two bacterial strains in tomato was evaluated by genetically labeling these strains with mCherry. Transformation to express the mCherry fluorescent protein was performed following recommendations of Ferreira et al. (2008). Briefly, plasmids were obtained from an overnight liquid culture (37° C, 220 rpm) of *E. coli* WM3064 containing Tn*7*::*mCherry* plasmid and *E. coli* DH5α containing helper plasmid pTNS3. Cultures were conducted in LB supplemented with spectinomycin and diaminopimelate (DAP). Plasmid extraction was performed using the Spin miniprep kit (Qiagen) following manufacturer recommendations. Bacterial strain competent cells were obtained according to the protocol of Ferreira et al. (2008). Strains in the exponential growing stage (O.D. 600 = 0.4 to 0.6) were washed three times in sucrose (300 mM). Bacterial strain competent cells (100 µL) were electroporated (Gene Pulser, BioRad -2.5kV, 25μF, 200Ω, 5s) with 500 ng of the Tn*7*::*mcherry* plasmid and 500 ng of helper plasmid pTNS3. After incubation (28° C, 220 rpm, 3h) the transformed cells were plated on LBA supplemented with spectinomycin (10 mg.L^{-1}). One colony was randomly selected and stored at -80° C in glycerol. Fluorescence of bacteria was evaluated by confocal analysis and plasmid insertion was verified by extraction DNA of bacterial strains thanks to a thermal shock (5 min at 100° C, 10 min on ice) and amplification of the mCherry gene with primers Tn*7*_2377F (5'-ACCGTAACACGCCACATCTT-3') and Tn*7*_3033R (5'-GCAATTGCCTGGTGCATACAA-3') applying the following thermal protocol: 5 min at 95° C, followed by 30 cycles of 45 seconds at 95° C, 30 seconds at 62° C, and 45 seconds at 72° C and a final extension during 10 min at 72° C. Tn*7* insertions take place at specific attachment sites located downstream of the coding region of *glmS* gene and have been demonstrated to incur no fitness penalty on the bacterial host (Lambertsen et al., 2004).

To evaluate the colonization capacity of two bacteria in tomato plants, seed coating was performed as described in section "Assessment of bacterial coating effects on seed vigor index" with the modification that the

bacterial strains were grown in LB medium supplemented with spectinomycin. Ten days after germination, the assessment of bacterial endophytic and epiphytic colonization within various plant parts was conducted by qPCR, colony counting assays, and confocal microscopy analysis. Three plants per bacterial strain were cut into leaves, shoots, and roots for direct analysis. Additionally, another set of three plants per treatment were surface-disinfected by submerging in a 5.25% sodium hypochlorite solution for six minutes, followed by three rinses in sterile water.

Development of a qPCR assay to quantify Tn*7* mutants in tomato

To quantify Tn*7*::mutant strains within various parts of *S. lycopersicum*, qPCRs were performed on plant DNA extracted using the CTAB method. Specific primers, F_Tn*7*_25511 and R_Tn*7*_2576 were designed to amplify the Tn*7* plasmid, confirmed for specificity through *in silico* analysis on Primer-Blast (NCBI) and PCR amplification of inoculated samples. Since the Tn*7* plasmid integrates at one position in the bacterial genome, it serves as a marker for bacterial colonization. Simultaneously, DNA quantification of *S. lycopersicum* was performed with primers TUB-F and TUB-R, targeting the β-tubulin reference gene (Lovdal and Lillo, 2009). The sequences of primers and the thermal cycling conditions are detailed in Table S-4. Standard curves were constructed by purifying PCR products for Tn*7* and β-tubulin amplicons using the SmartPure PCR kit (Eurogentec). DNA concentrations were measured using the Qubit HS assay kit (ThermoFisher Scientific), adjusted to 10^{10} copies and serially diluted to create calibration curves correlating known DNA quantities to cycle threshold (Ct) values. qPCR mixes contained 2×Takyon SYBR MasterMix dTTP (Eurogentec), 500 nM of each primer, and 2 µL of DNA template, in a 20 µL reaction volume. Amplifications were performed on a CFX96 thermocycler (Bio-Rad) in duplicate. Conditions for bacterial quantification were: initial denaturation at 95° C for 10 minutes, followed by 40 cycles at 95° C for 15s, 54° C for 15s, and 72° C for 15s. For TUB amplification, the annealing temperature was modified to 60° C. The β-tubulin gene was confirmed to be in single-copy in the genome of *S. lycopersicum*. The number of β-tubulin copies was approximately 975 copies per nanogram of DNA as the genome size of *S. lycopersicum* is approximately 950 million bp. This allowed calculation of bacterial quantity per gram of plant DNA using the equation:

$$\text{Bacteria(ng of plant DNA)}^{-1} = \left(\frac{\text{Number of Tn}7\text{ copies}}{\left(\frac{\text{Number of TUB copies}}{975} \right)} \right)$$

For qPCR quantification, three plants were surface disinfected, and three others were not, in order to quantify epiphytic and endophytic bacterial colonization.

Colony-counting assay

The colony-counting assay was performed to confirm the presence of viable inoculated bacteria in the different organs of the plant. As for qPCR quantification, three plants were surface disinfected, and three others were not in order to quantify epiphytic and endophytic bacterial colonization. Roots, shoots, and leaves were separated, weighed, and crushed in PBS. Then, samples of each plant organ were diluted up to 10^{-6} and 100 µL from each dilution was spread in triplicate on LBA supplemented with spectinomycin (10 mg.L^{-1}) and incubated at 28° C for 24 hours.

Localization of mCherry-labelled strains in tomato with confocal laser scanning microscopy (CLSM) analysis

In parallel, plant parts were collected, and the roots, shoots, and leaves were transversally/vertically cut and distributed on a microscopic slide with Fluoromount-GTM (Thermo Fisher Scientific) solution. No surface disinfection was done for this observation. Ten-day seedling roots were also immersed for three hours in a bacterial solution of C1M8::mCherry and C1M39::mCherry adjusted in PBS at 10^8 CFU/mL to determine whether the bacterial strains isolated from the seeds were able to move through the plant vascular tissues. Observations were performed using a confocal microscope (Leica Stellaris confocal 8 flacon laser scanning microscope) with the objective HC PL APO CS2 63x/1.20 water following their collection at the platform IMABIOL (UCLouvain, Belgium). For the mCherry signal, the red emission was stimulated at 565 nm.

3.5. Statistical analysis

Statistical analysis was performed using RStudio version 4.3.0. The effect of bacterial coating on the seed vigor index was assessed for statistical significance between the treated groups and the control group using Student's t-test ($p \leq 0.05$), after confirming the normality of residuals. For differences in the colonization of various plant organs by mCherry-tagged mutants, the Wilcoxon test was employed, as the residuals did not meet the normality assumption.

4. Results

4.1. Identification of the seed-transmitted endophytic bacteria by metabarcoding

The sterility of the hydroponic culture system was confirmed as no bacterial growth was observed on the media inoculated with the hydroponic solution devoid of plants. Additionally, no amplification was detected following the 16S rRNA gene amplification from samples of the hydroponic solution without plants.

Despite the use of universal PNAs (Lundberg et al., 2013), the amplifications revealed fragments of different sizes (between 3000 and 6000 bp), suggesting that one or more plant DNA regions were co-amplified along with the bacterial *rrn* operon. Analysis of Illumina sequencing results confirmed that the percentage of reads associated with tomato was quite high (between 40 and 50%), and a large proportion of these reads were assigned to a chromosome 11 region in *S. lycopersicum* (Figure S-1, Table S-5, S-6). The transfer of organelle DNA fragments to the nuclear genome is frequently observed in eukaryotes and could explain the presence of a sequence close to the chloroplast 16S gene in a chromosome (Yoshida et al., 2014).

To drastically reduce the proportion of plant-origin reads, we designed a new PNA clamp, PNA-chr11 (Table V-1), by aligning the plant-amplified sequences with the V1-V3 region of 16S rRNA bacteria sequences. To match completely with all the sequences amplified, two bases of the PNA were degenerated. *In silico* analysis revealed that only genome of *S. lycopersicum* and other plants species aligned with the sequence proposed. Sequencing of plant endophytic communities amplified with the PNA-chr11 revealed that the use of the new PNA suppressed almost completely tomato genome amplification with a proportion of reads attributed to plant of 0.14% compared to 60.2% without it (Figure S-1).

To capture the results of the infection dynamics throughout plant development from seed to shoots and roots, HTS-based analyses were performed using DNA extracted from surface-disinfected seeds, shoots, and roots of plants grown under sterile conditions. To ensure data robustness and reliability, replicate samples were done: three pools of seeds, six root parts, and six shoots were collected. HTS was performed on 16S-ITS-23S amplicons, resulting in 682,539 high-quality duplex reads (Figure S-2). After discarding chimeras, singletons, 675,222 reads remained with an average abundance of 39,719 reads per sample and a standard deviation of 24,417 reads. The bioinformatic reconstruction of

Table V-1: Sequence and characteristics of a new PNA-chr11 clamp designed to block amplification of the chromosome 11 of *S. lycopersicum* var. Moneymaker.

Name	Sequence	Length	Tm	Purine (%)	GC content (%)
PNA-chr11	CTGCTAATACCYCGKAGGCTGA	22	84	45.5	54.5

the bacterial communities identified a total of 53 distinct representative sequences. To simplify the lecture, we will talk about OTUs.

13 OTUs were identified within the seeds, comprising the genera *Ralstonia*, *Bradyrhizobium*, *Sphingomonas*, *Paucibacter*, *Microbacterium*, *Stenotrophomonas*, *Lawsonella*, *Nocardioides*, and *Mucilaginibacter*. Of these, six genera: *Ralstonia*, *Bradyrhizobium*, *Sphingomonas*, *Paucibacter*, *Microbacterium*, *Stenotrophomonas* were ubiquitously present across all examined plant parts, indicating a widespread distribution from seeds in the phytobiome (Figure V-2, V-3). These genera were defined as core seed-transmitted endophytic bacterial community. In contrast, three genera: *Lawsonella*, *Nocardioides*, *Mucilaginibacter* were exclusively identified in the seeds, suggesting a unique ecological niche or specific environmental conditions favorable to these genera within the seed microhabitat. Additionally, 28 other genera were identified in roots and shoots with 12 of these being common to both roots and shoots (Figure V-3). The prevalent bacterial phylum identified in tomato was Proteobacteria, with *Ralstonia pickettii* being the most abundant species, comprising 98.9%, 91.3%, and 34.7% of the bacterial community in the seeds, the shoots, and the roots respectively (Figure V-2).

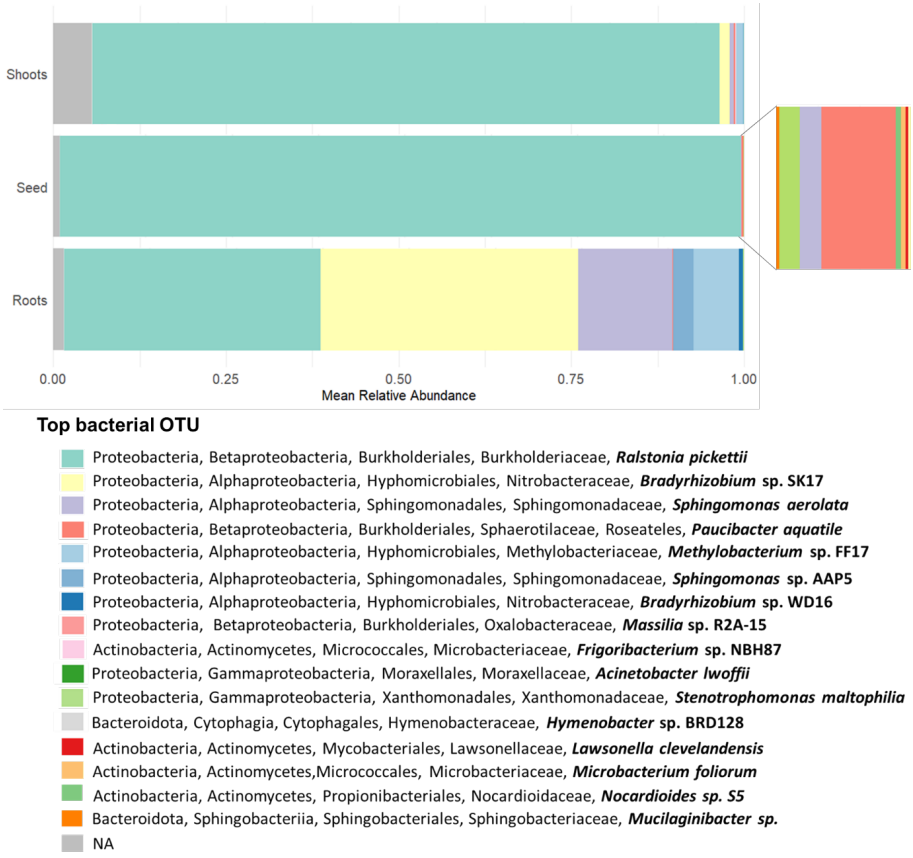

Figure V-2: Composition of bacterial species in seeds, roots and shoots of *S. lycopersicum* var. Moneymaker as detected by HTS sequencing of the 16S-ITS-23S *rrn* operon (4,500 bp).
To have a complete picture of the diversity, the results obtained for all roots or shoots samples were merged, regardless of the disinfection status. Representative sequences were identified as bacterial strains based on the percentage of identification. These bacterial strains are highlighted in bold. Three pools of tomato seeds, six shoots and six roots parts were sequenced individually

Comparison of the bacterial composition associated with seeds, shoots and roots was performed using Observed features, Shannon, Simpson and InvSimpson diversity indexes. This analysis revealed significant differences with an increase in diversity in shoots and roots compared with seeds (Figure S-3).

Beta diversity, analyzed with a Bray-Curtis distance matrix, underlined dissimilarity between bacterial endophytic communities present in shoots, roots, and seeds, confirmed by a Permutational Multivariate Analysis of Variance (PERMANOVA) analysis (p-value = 0.0011)

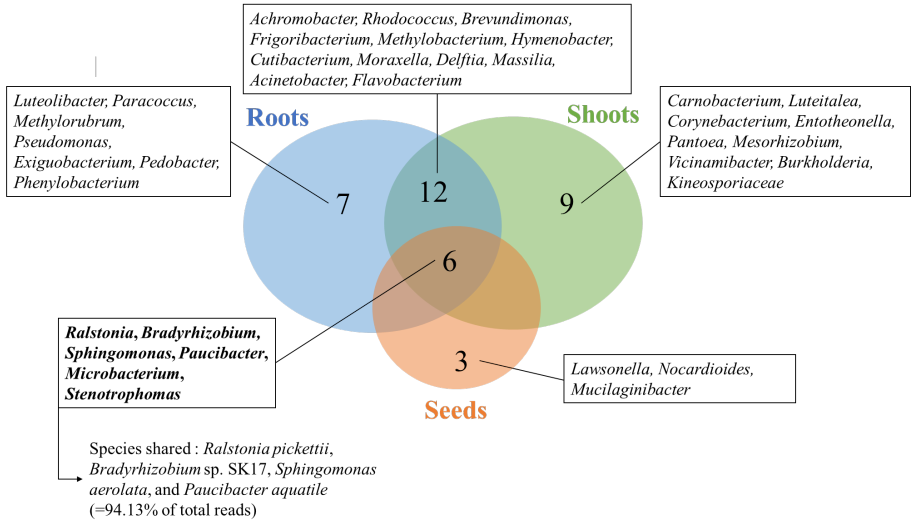

Figure V-3: Venn diagram representing vertically-transmitted endophytic bacterial genera identified in tomato seeds and in roots and shoots of tomato grown in sterile conditions for three weeks.
The percentage of reads common to all plant parts is 94.13%.

(Figure S-4). However, the observed dissimilarities are mainly due to rare OTUs. Indeed, 635,591 reads of bacterial endophytes were shared between seeds, roots, and shoots, representing 94.13% of total reads (93.25% of reads are shared in shoots, 93.06% in roots, and 99.34% in seeds).

In order to investigate the type (endo- or epiphytic) of colonization of endophytic seed-borne, we compared the bacterial composition of plant parts with and without surface-disinfection. For the roots, the genera *Luteolibacter*, *Paracoccus*, *Hymenobacter*, and *Moraxella* seemed to be epiphytic genera as they were only found in non-disinfected roots. In parallel, a higher number of genera were epiphytes in shoots including *Carnobacterium*, *Rhodococcus*, *Delftia*, *Flavobacterium*, *Luteitalea*, *Corynebacterium*, *Stenotrophomonas*, *Kineosporiaceae*, and *Brevundimonas* Figure V-4.

The seed-transmitted core microbiota composed of *Ralstonia*, *Sphingomonas*, *Bradyrhizobium*, *Roseateles*, and *Microbacterium* genera was found in roots and shoots regardless of their surface-disinfection status, confirming their endophytic behavior. However, interestingly, *Stenotrophomonas*, identified in seed, was found as an endophyte in roots but as an epiphyte in shoots. Additionally, some genera were found to be endophytes only in one plant part such as *Delftia*, *Brevundimonas*, *Cutibacterium*, *Achromobacter*, *Rhodococcus* in roots and *Hymenobacter*, *Acine-*

tobacter in shoots. Surprisingly, eight bacterial genera were uniquely identified in surface-disinfected roots and another eight exclusively in shoots Figure V-4.

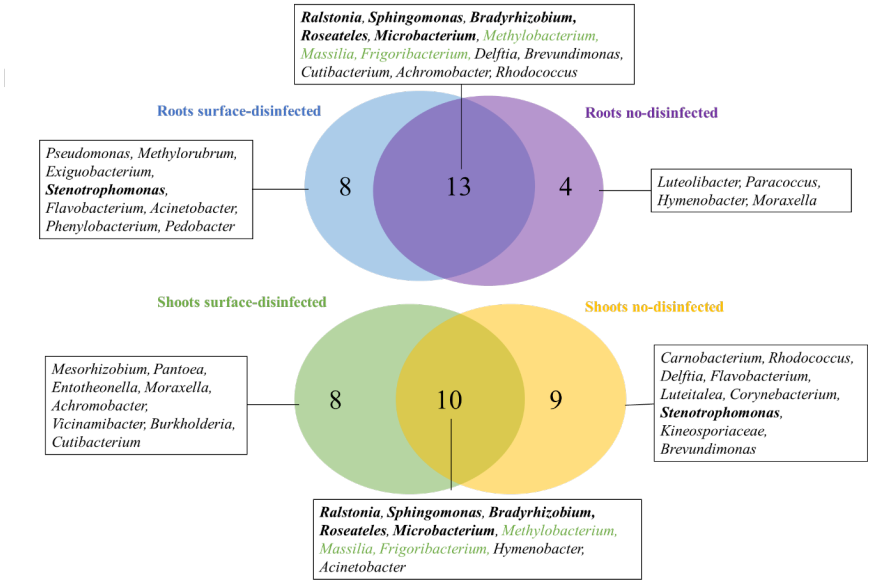

Figure V-4: Venn diagram representing vertically-transmitted endophytic bacterial genera found in roots (blue and purple) and shoots (green and yellow) of *S. lycopersicum* grown in sterile conditions for three weeks, regardless of their surface-disinfection status.
Genera shared between seeds, shoots, and roots are highlighted in ***bold****. Genera shared between shoots and roots but not identified in seeds are in green.*

4.2. Identification of seed-transmitted endophytic bacteria by culture-dependent method

Four different nutrient-rich levels of media were used during bacteria isolation to obtain diverse taxonomic strains of endophytes. In total, 111 bacterial strains were successfully isolated and purified with 18 strains coming from seeds, 23 from roots, 40 from shoots, and 30 from the hydroponic solution of *S. lycopersicum* var. Moneymaker. The 16S rRNA gene sequencing enabled their classification into 23 genera from four phyla: Firmicutes (52.25%), Actinobacteria (25.26%), Proteobacteria (21.62%) [Alphaproteobacteria (9%), Betaprotebacteria (4.51%) and Gammaproteobacteria (8.11%) classes] and Bacteroidetes (0.87%). *Bacilli*, the most abundant class, was composed of the genus *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Rummeliibacillus*, *Peribacillus*, *Neobacillus*, *Aerococcus*, *Staphylococcus*, and *Fredinandcohnia*.

Figure V-5 provides a summary of the isolated bacterial species and their origins. The highest abundance and diversity of species were observed in the shoots of the plant. Seeds predominantly contained bacterial endophytes from the Firmicute phylum, constituting 94% of the total. In the shoots, endophytic bacteria were divided into four phyla, with Actinobacteria being the most prevalent (42.5%), followed by Proteobacteria (30%), including representatives of the Alpha, Beta, and Gamma classes, Firmicutes (25%), and Bacteroidetes (2.5%). The root-associated endophytes were primarily Firmicutes (60.9%), with Proteobacteria (21.7%) and Actinobacteria (17.4%) also represented. In the hydroponic solution, under sterile conditions, the endophytes likely originated from the seeds and roots, with the majority belonging to Firmicutes (56.7%), followed by Proteobacteria (23.3%), and Actinobacteria (20%).

The RAPD analysis revealed two isolates of *Bacillus zanthoxyli* (C1M28 and C1M38) and two isolates of *Brevibacillus centroporus* (C1 25 and C1 26) were found to be genetically and morphologically identical.

PGP traits of culturable seed-transmitted bacterial endophytes

Isolated tomato endophytes were first screened for their ability to tolerate osmotic stress. Among 111 isolates, 67 bacterial isolates were able to grow in LB supplemented with PEG 6000 (10% w/v) having a growth ratio between 0.5 and 1. Of the 67 bacterial strains selected Figure V-5, 14 were identified as highly tolerant to drought with an inhibition rate below 25%. Most of them belong to the Firmicute phyla (78,6%) including the species *Bacillus velezensis*, *B. tequilensis*, *B safensis*, *B. zanthoxyli*, *Perbacillus frigoritolerans* and *Brevibacillus centroporus*. In the phyla Actinobacteria, one species, *Micrococcus aloeverae*, and in the Proteobacteria, the species *Pseudomonas protegens* also displayed a high tolerance to osmotic stress. Twenty-six bacterial isolates had an inhibition rate between 25% and 50% and were considered drought-tolerant, mostly within the Firmicute phyla (Figure V-5).

Among the 67 isolates, all but two, produced IAA in the presence of the tryptophane precursor, the IAA production ranging from 0.32 to 15.65 $\mu g.mL^{-1}$. Four bacterial isolates were identified to produce higher IAA content: *Microbacterium arabinogalactanolyticum* (C2 4) with 15.65 $\mu g.mL^{-1}$, *B. tequilensis* (C2 20) with 10.72 $\mu g.mL^{-1}$, *P. protegens* (C1 M8) with 6.20 $\mu g.mL^{-1}$ and *B. velezensis* (C1 45.1) with 6.12 $\mu g.mL^{-1}$.

Thirty out of the 67 tested isolates were shown to be able to solubilize phosphate *in vitro* and most of them belonged to the genera *Bacillus*,

Paraburkholderia and *Pseudomonas*.

Siderophore production ability was evidenced for 32 isolates with the higher production observed for bacteria belonging to genera *Bacillus*, *Paraburkholderia* and *Pseudomonas*.

The production of EPS was observed for 33 isolates in the genera *Bacillus*, *Peribacillus*, *Neobacillus*, *Rummeliibacillus*, *Brevibacillus*, *Microbacterium*, *Micrococcus*, *Kocuria*, *Brucella*, *Pseudomonas*, *Paraburkholderia*.

After PGP tests *in vitro*, a selection was made for *in planta* test. The main selection criteria included first a high tolerance to osmotic stress and the production of EPS and secondly, the ability to produce auxin, solubilize phosphate and synthesize siderophores. When several strains belonged to the same species, only one was selected. However, an exception was made for the genus *Pseudomonas*, two strains were selected due to their distinct PGP characteristics and because the 16S rRNA identification method offers poorer resolution within this genus (Lauritsen et al., 2021). Initially, one strain of *B. velezensis* was selected, but this was later discarded due to difficulties with liquid culture. The nine bacteria selected were: *B. safensis* (C1 5b), *P. frigoritolerans* (C2 12), *B. wiedmannii* (C2 52b), *P. graminis* (C2 30b), *B. zanthoxyli* (C1 M38), *P. protegens* (C1 M8 and C1 M39), *R. stabekisii* (C1 47.2) and *B. tequilensis* (C2 20) Figure V-5.

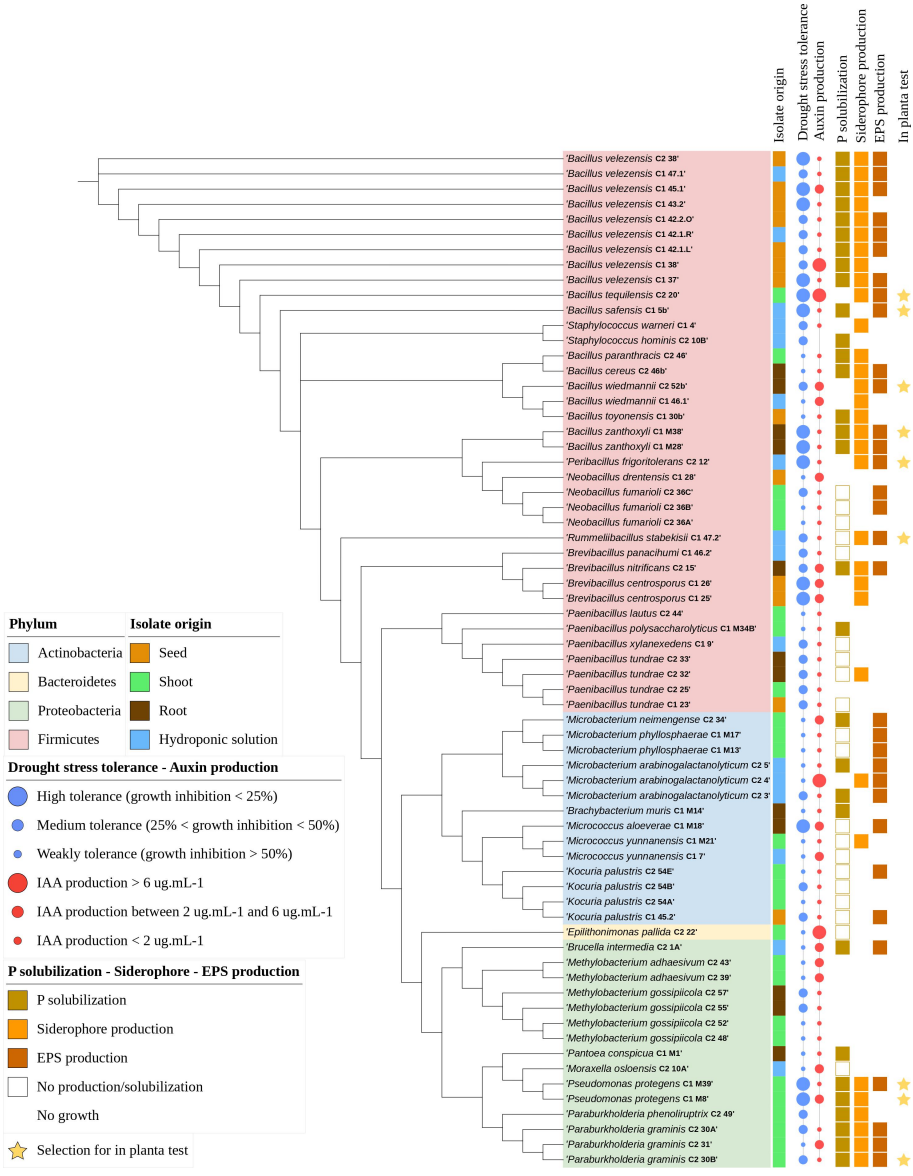

Figure V-5: Phylogenetic tree and PGP traits of the cultivable endophytic bacterial strains of *S. lycopersicum* (var. Moneymaker) isolated from seed, shoots, roots and in hydroponic solution

Circles are used for quantitative results. For drought stress, small, medium, and large circles represent bacterial strains with no or weak tolerance (growth inhibition $> 50\%$*), medium tolerance (*$25\% <$ *growth inhibition* $< 50\%$*), and high tolerance (growth inhibition* $< 25\%$*) to osmotic stress (PEG 10% w/v), respectively. For auxin production, small, medium, and large circles represent IAA production of* $< 2\,\mu g.mL^{-1}$*, between* $2\,\mu g.mL^{-1}$ *and* $6\,\mu g.mL^{-1}$*, and higher than* $6\,\mu g.mL^{-1}$*, respectively.*

Phosphate solubilization, siderophore, and exopolysaccharide production are represented as qualitative results: no square indicates that the bacterial strain does not grow on the selective media, empty and full squares indicate a negative or a positive result, respectively. Bacterial strains selected for in planta tests are marked with a star.

Effect of bacterial strains on seed germination and seedling growth under osmotic stress conditions

Seed coating with three of the nine selected bacterial strains led to a significant enhancement of the seedling vigor index, obtained through germination percentage, shoot, and root lengths (Table S-7). Seeds coated with bacterial strains *Rummeliibacillus stabekisii* (C1 47.2) and *Peribacillus frigoritolerans* (C2 12) led to more vigored seedlings under both non-stress (0 MPa) and osmotic stress conditions (-0.2 MPa). Conversely, seed coating with *Pseudomonas protegens* (C1 M8) exhibited an increased vigor index solely under non-stress conditions (Figure V-6).

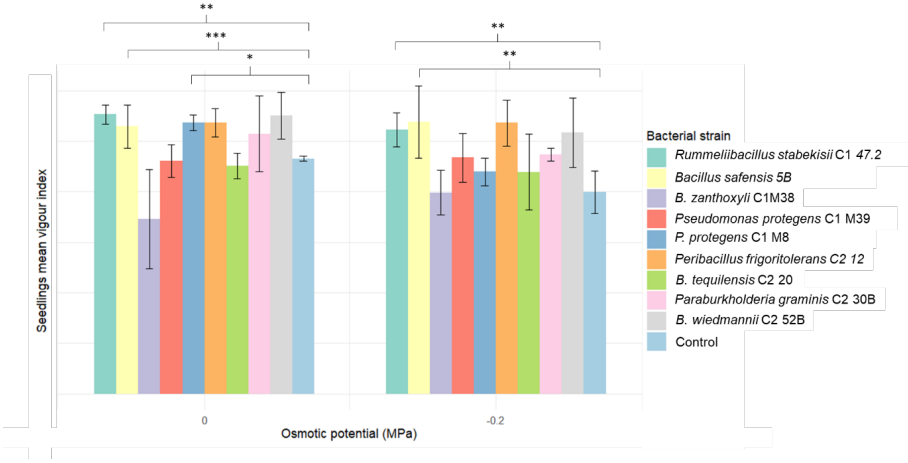

Figure V-6: Seedling vigor index of tomato seed coating with bacterial strains under non-stress (0 MPa) and osmotic stress conditions (-0.2 MPa) (10 DAS).
*The significance of the data is assessed using a Student's t-test, where * represents a p-value < 0.1; **: 0.01 < p-value < 0.05; ***: p-value < 0.01.*

4.3. Confirmation of the endophytic behavior of isolated bacteria with mCherry mutants

The two *Pseudomonas* strains, C1M8 and C1M39, were selected for transformation due to the relative ease of creating mutants in Gram-negative bacteria and because this genus was identified in both HTS and

media isolation. The mCherry-tagged *P. protegens* C1M8 and C1M39 strains exhibited similar growth patterns and cell morphologies of the corresponding wild-type strains on LBA without spectinomycin. However, they displayed fluorescence when exposed to red light. The insertion of the plasmid in these strains was confirmed by PCR. Using the confocal microscope, we determined that the cells from both strains (C1M8 and C1M39) remained alive and exhibited fluorescence in a free-living state (Figure S-5).

Ten days after the germination of seed coated, these mCherry-marked C1M8 and C1M39 strains were quantified in different plant organ tissues by colony counting and qPCR. During the germination phase, bacterial strains proliferate and establish colonization within roots and shoots in both internal tissues and surface. It is mainly about endophytic colonization, as no significant difference appeared in colonization of surface-disinfected and non-disinfected plants analyzed by colony counting for C1M8 and C1M39 (p-value > 0.05). Notably, both bacterial strains demonstrate similar colonization patterns in plant parts (p-value > 0.05) with a trend to favor endophytic presence within the leaves, followed by the roots and subsequently the shoots (Figure V-7), although without statistical significance (Table S-8,Figure S-6). Specifically, strain C1M8 exhibited heightened abundance within the leaves, registering concentrations of 1.71×10^{10} CFU of C1M8 per gram of fresh weight in non-disinfected plants (n=5) and 1.67×10^{7} CFU of C1M8 per gram of fresh weight in disinfected plants (n=5). Conversely, strain C1M9 appears to predominantly colonize the surface of roots and stems, with concentrations of 1.19×10^{10} and 4.46×10^{9} CFU per gram of fresh weight (n=5), respectively (Figure V-7) (Table S-8).

The qPCR assay was performed in addition to the colony counting and CLSM analysis (Figure V-7) in the endophytic and epiphytic colonization process. The primers used in the Tn*7* qPCR showed an efficiency of 0.997 based on the slope and the line equation of the standard curve (slope = -3.328, R2 = 0.99) (Figure S-7) and no match to the plant DNA content (genome, plastid, mitochondria of *S. lycopersicum*) was observed in primer-blast analysis. No difference was observed in the colonization pattern of both bacterial strains or in colonization of plant parts for each bacterial strain confirming the results observed by colony counting (Figure V-7).

The localization of mCherry-marked *P. protegens* C1M39 and C1M8 within the plant parts was clarified by CLSM. In plants grown after seed coating, the bacteria were mainly found in apoplast spaces in all plant parts (Figure V-8 B, C). Interestingly, the mCherry-marked C1M39 was observed inside a stomata as highlighted in Figure V-8 C. After roots were immersed in the bacterial solution, the presence of both bacterial strains was visible in vascular tissues, demonstrating the endophytic

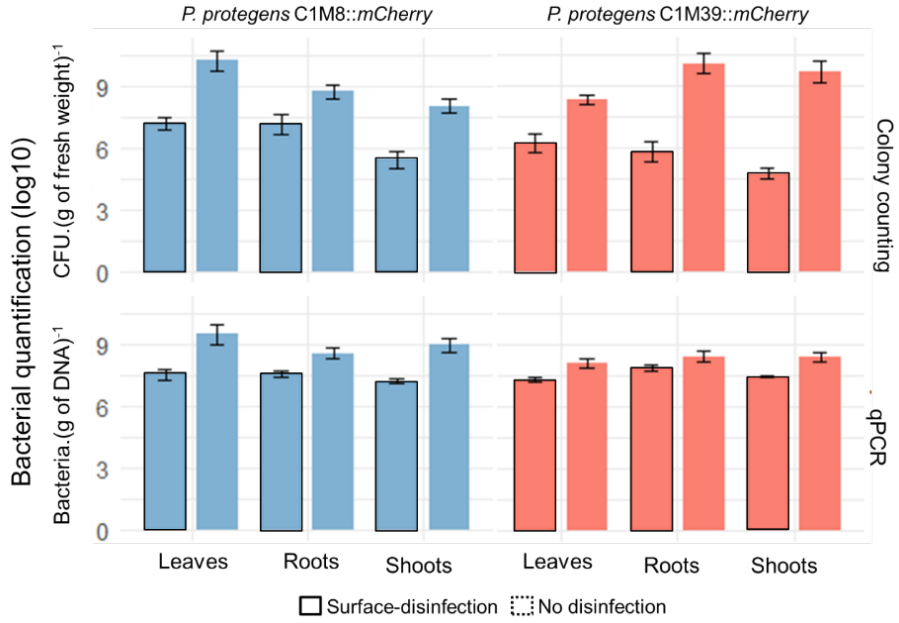

Figure V-7: Quantification through colony counting on media and qPCR of mCherry-marked *P. protegens* C1M8 and C1M39 in roots, shoots and leaves of non-disinfected and disinfected tomato plants

character of these bacteria (Figure V-8 A, F). As observed in Figure V-8 E), bacteria penetrated the apical regions of roots to enter the plant vascular tissues (Figure V-8F), as well as the apoplast (Figure V-8D) and symplast (Figure V-8C).

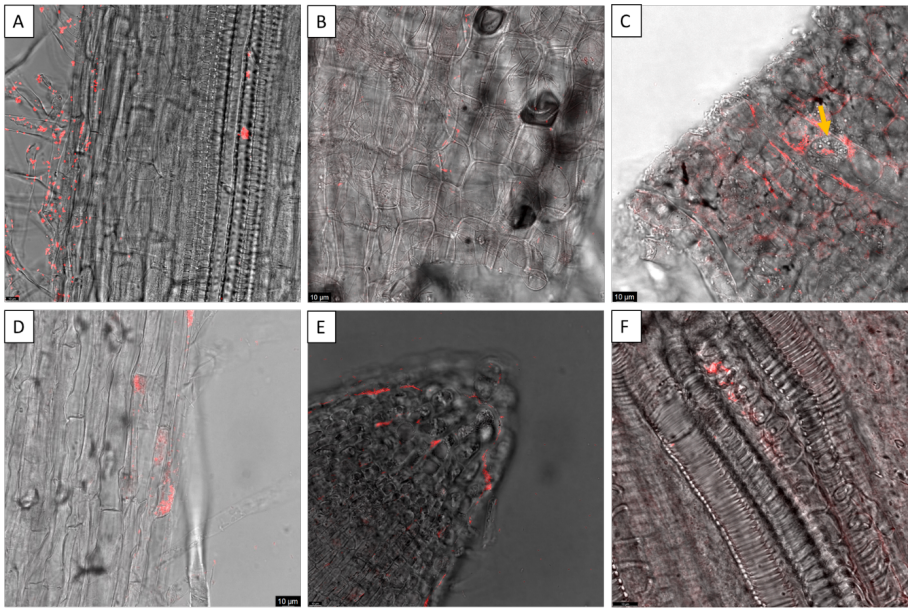

Figure V-8: Confocal laser scanning microscopy analysis showing *P. protegens* C1M39::*mCherry* (A, B, C) and C1M8::*mCherry* (D, E, F) in plants.

(A) represents roots of ten-days seedlings that were deep in C1M39:: mCherry suspensions adjusted to 10^8 *CFU/mL for three hours. (B) and (C): roots and leaves of tomato seedlings grown under sterile conditions for 10 days from seeds coated with C1M39::mCherry. Arrow indicate stomata. Roots of ten-day plantlets were immersed into C1M8::mCherry suspension adjusted to* 10^8 *CFU/mL for three hours, (D) and (E) represent roots and root cap respectively and (F) shoots of tomato after root immersion.*

5. Discussion

In this experiment, we deepen our knowledge on seed-transmitted bacterial endophytic communities of *S. lycopersicum* var. Moneymaker using a sterile hydroponic culture system where plants were grown for four weeks. The communities were characterized through a polyphasic approach, combining HTS and isolation, and phylogenetic classification of culturable bacteria.

5.1. Key species in tomato plant bacteriome and colonization specificity

To accurately characterize the endophytic bacteria using metabarcoding, it was crucial to minimize bias in tomato sequence amplifications caused by chloroplast and mitochondrial DNA, while maximizing the recovery of reads from endophytic bacteria (Fitzpatrick et al., 2018b). We addressed this challenge by designing a specialized PNA clamp tailored to the tomato variety Moneymaker, which significantly reduced the proportion of tomato reads from 60.2% to 0.14% of the total reads. By combining this advanced method with sequencing of the 16S-ITS-23S region of the *rrn* operon, we achieved species-level identification of endophytic communities within seeds, roots, and shoots.

Alpha-diversity indices of endophytic bacterial communities (Figure S-3) highlight that the core microbiota increased upon seed germination in roots and shoots. This result suggests that rare bacterial species were able to thrive increasing their relative abundance (Abdelfattah et al., 2021; Faddetta et al., 2021). The development of these rare species could explain differences in beta diversity (Figure S-4), as the shared OTU between seeds, shoots, and roots represented 94% of the reads. Intriguingly, certain taxa, observed in low quantities in the shoots and roots of the developing seedling, were not initially detected in the seed. This discrepancy can be explained by several interrelated factors. Biologically and statistically, variability among individual seeds might lead to differences in the microbial communities present initially in the seeds compared to those emerging in the seedlings. Additionally, it is possible that some taxa, initially undetectable, could have proliferated after being transmitted to the growing seedling. Furthermore, dominant genera such as *Ralstonia*, in this experiment, might obscure the detection of less abundant taxa. This hypothesis is supported by the fact that surface disinfection enables the detection of genera that are not observable without such treatment. This highlights the importance of complementing culture-dependent approaches with metabarconding to obtain the most "complete picture" of bacterial diversity. Finally, the possibility of minor experimental contamination also cannot be ruled out. However, the sterility of the system was thoroughly validated, making this hypothesis unlikely. Similarly, Faddetta et al. (2021) reported that the shoots of 15-day-old *Citrus limon* plantlets grown under sterile conditions *in vitro* on Murashige and Skoog (MS) solidified medium exhibited increased microbial diversity, including the detection of new genera do not present in the original seed communities. Abdelfattah et al. (2021) observed the transmission of endophytes from seeds to shoots and roots of oak seedlings grown in a microbe-free environment, where aerial parts and roots were separated. They also noted that some taxa, found in relatively low abundance in the phyllosphere and roots, were not initially present in the seeds.

When comparing epiphytic and endophytic colonization of bacteria, distinct distributions within roots and shoots were observed. For instance, the genus *Stenotrophomonas* demonstrated niche differentiation, with endophytic colonization in roots and epiphytic colonization in shoots. These variations may result from a combination of life-history traits and plant regulatory factors that guide bacterial migration to specific plant parts (Abdelfattah et al., 2021). It is not surprising to find epiphytic colonization by seed-borne endophytes, as these bacteria can emerge during germination and interact with soil microorganisms (Hardoim et al., 2012). However, many studies on the transmission of endophytic bacteria from seeds often overlook these diverse colonization modes (Bergna et al., 2018; Faddetta et al., 2021).

Our research focused on characterizing the core microbiome, specifically identifying bacteria that consistently appear in both the seed and the seedling. Given the high percentage of shared reads between plant organs, our findings likely represent the core microbiota transmitted from the seed to roots and shoots including *Ralstonia pickettii*, *Bradyrhizobium* sp. SK17, *Sphingomonas aerolata*, and *Paucibacter aquatile*. Interestingly, *Ralstonia* and *Sphingomonas* were also central to the seed-borne microbiota of the tomato cultivars 'Arka Vikas' and 'Arka Abha', suggesting that certain genera may be common to the core microbiota, regardless of the cultivar or the experimental conditions (Shaik and Thomas, 2019). *R. pickettii*, present in high abundance in all plant parts, is an oligotroph, thriving in nutrient-scarce environments and various strains of *Ralstonia* are resistant to contamination by glyphosate herbicides or heavy metals, like manganese (Huang et al., 2018; Kuklinsky-Sobral et al., 2005). Additionally, another strain from the tomato rhizosphere was shown to act as a biocontrol agent, offering protection against *R. solanacearum* (Wei et al., 2013). The presence of nitrogen-fixing *Bradyrhizobium* in the primary microbiota of hydroponically grown tomato plants aligns with expectations, given its diazotrophic endophytic properties (Terakado-Tonooka et al., 2023). These characteristics are especially beneficial in hydroponic systems with controlled oxygen levels that favor nitrogen fixers (Smercina et al., 2019). Other studies revealed that the core microbiota of tomato generally comprises a predominance of Proteobacteria, as well as Bacteroidetes, Actinobacteria, and Firmicutes (Lopez et al., 2020; Tian et al., 2017).

The near-complete absence of Firmicutes in our metabarcoding results is particularly unexpected given that *Bacillus* spp. are considered as the most common seed-associated bacteria across plant species (Frank et al., 2017; Nelson, 2018; Truyens et al., 2015). The non-occurrence of the *Bacillus* genera may be attributed to two hypotheses. First, *Bacillus* spp. might not establish well in the roots under hydroponic conditions due to the environment not being adapted for their typical growth requirements. Indeed, such conditions are better met in soil as most of

the *Bacillus* spp. are aerobic or facultative aerobic bacteria (Nakano and Zuber, 1990). This is supported by results from dos Santos et al. (2021), where Firmicutes were absent from the roots of hydroponically grown cucumbers. Similarly, the roots of tomatoes grown under hydroponic conditions were also devoid of Firmicutes in the experiment of Sato et al. (2023). However, it is still surprising not to find this genus in the seeds and shoots. Second, the method of analysis could influence the proportions of *Bacillus* observed. Shaik and Thomas (2019) showed a predominance of Firmicutes in culture-based methods, whereas Proteobacteria were more dominant in metabarcoding studies (65.7-69.6% OTU). Additionally, it is crucial to acknowledge that the methodological approach employed in DNA extraction and sequencing could significantly influence our ability to detect *Bacillus* spp. Gram-positive bacteria such as these bacteria have particularly robust cell walls that can hinder effective DNA extraction, and their propensity to secrete DNases may degrade DNA, complicating its sequencing. Indeed, our reliance on longer amplicons exceeding 4 kb for sequencing may pose challenges in detecting partially degraded DNA. This phenomenon was notably observed in a recent study where *Bacillus* was detectable using Illumina technology but was missed in nanopore sequencing using two different sets of primers targeting the full operon (Dubois, unpublished data). This second hypothesis was supported by our study of cultivable seed-transmitted endophytic bacteria, which revealed predominantly bacterial isolates belonging to Firmicutes, Actinobacteria, and Proteobacteria phyla. This result is consistent with those typically reported as cultivable endophytes in the literature (Truyens et al., 2015).

5.2. Comparison of endophytic communities identified through metabarcoding and culture-dependent methods

In this study, we used both culture-dependent and metabarcoding approaches, each with distinct advantages and limitations. Traditional microbiological methods allowed for the isolation and characterization of phenotypical traits of culturable bacteria, while metabarcoding offered a comprehensive view of the microbial community, including non-culturable members. These methods are complementary: metabarcoding provides a broad overview, and microbiological methods enable detailed studies of specific strains. Notably, the majority of isolated strains did not belong to the most abundant genera revealed by the metabarcoding analysis, only 20 isolates belonged to genera identified using metabarcoding. This discrepancy is commonly observed and has been previously reported (Faddetta et al., 2021; Zeyaullah et al., 2009). It can be attributed to the limitations of culture media, which cannot encompass the entire microbiota. While metabarcoding provides a global view of microbial communities, culture-dependent methods can isolate specific

strains below the detection limit of metabarcoding approaches, highlighting their complementary nature. Moreover, an experimental bias in microbiota characterization of the 16S-ITS-23S *rrn* operon cannot be completely excluded (Hugerth and Andersson, 2017), but it is unlikely knowing that the primers used were confirmed in this study as universal by comparison with three other commonly used primer pairs.

5.3. PGPB in seed-transmitted bacteria

The culture-dependent approach allowed us to explore the properties and functions of a significant portion of the seed microbiota. This functional aspect is crucial, as it provides insights into the roles these endophytes play in plant health and development, emphasizing their importance beyond mere presence within the plant. Our results underscore the importance of seed-transmitted bacteria in enhancing plant tolerance to abiotic stress. Most isolates were capable of producing IAA and solubilizing phosphate, while approximately half could produce siderophores and EPS. These functions can facilitate plant growth directly, indirectly, or synergistically (Cherif et al., 2015; Rolli et al., 2015; Vurukonda et al., 2016). For instance, bacterial auxin production has been shown to enhance plant yield under drought stress (Shahzad et al., 2018), playing a critical role in regulating plant responses to salinity and drought by influencing gene expression through auxin response factors (ARFs). ARFs regulate soluble sugar content, promote root development, and maintain chlorophyll levels, helping plants adapt to these environmental stresses (Verma et al., 2022). Furthermore, phosphate solubilization can mitigate the negative effects of drought by increasing root biomass and enhancing root hydraulic conductance under drought conditions (Abbasi, 2023). Siderophore-producing microbes alleviate iron deficiency and improve the physiological and biochemical processes of crops in saline soils, which are similar to osmotic stress conditions (Singh et al., 2022). Additionally, EPS-producing bacteria help maintain higher soil moisture content, protect plant roots from desiccation, and facilitate microbial aggregation, thereby enhancing plant-microbe interactions (Naseem et al., 2018). Notably, more than half of the isolates were able to grow under osmotic stress (10% PEG), suggesting that these bacteria could remain active and express their plant growth-promoting traits even under such stress conditions (Chen et al., 2017).

Seed coating trials further confirmed the value of seed-transmitted endophytic bacteria. Selected strains *P. frigoritolerans* C2 12 and *R. stabekisii* C1 47.2 enhanced root and shoot growth under both stress and non-stress conditions, increasing plant vigor. These strains demonstrated osmotic stress tolerance and significant EPS and siderophore production in PGP tests. Additionally, *P. protegens* C1 M8, which can solubilize phosphate and produce siderophores, enhanced plant vigor

only under non-stress conditions, likely due to its lack of EPS production, a key factor in plant drought resilience (Naseem et al., 2018). These findings highlight that the plant growth-promoting activity of bacteria can be either stress-dependent or stress-independent (Rolli et al., 2015). Further supporting our findings, previous studies have highlighted the beneficial roles of similar strains. *P. frigoritolerans* T7-IITJ has been reported to induce plant-growth promoting genes in Arabidopsis thaliana under osmotic stress (Marik et al., 2024), and *P. frigoritolerans* A70 has been shown to enhance the performance of *Medicago polymorpha* under osmotic stress (Gil et al., 2023). Similarly, *R. stabekisii* is known as a halotolerant species with biomineralization potential (Mudgil et al., 2018).

5.4. Colonization abilities of seed-transmitted endophytic bacteria

In addition to their PGP traits, efficient colonization by inoculated bacteria is a crucial step in plant-endophyte interactions (Compant et al., 2005). The colonization ability of *P. protegens* C1M8::mCherry and C1M39::mCherry mutants was confirmed when applied through seed coating or root dipping. When seeds were coated, the bacteria were predominantly observed in apoplast spaces rich in nutrients (Hardoim et al., 2015) but not plant vessels. On mature ungerminated seeds, bacteria colonized the seed coat (Barret et al., 2016), suggesting migration with plant development rather than through vessels. Conversely, root-dipping led to colonization on the root surface, penetrating at root tips and lateral root cracks, and spreading to the apoplast, symplast, and vascular tissue. Bacteria were observed in shoots within three hours, confirming propagation through vascular tissues, similar to *Burkholderia phytofirmans* PsJN in grapevine plantlets (Compant et al., 2005). The mode of inoculation influenced colonization patterns: seed coating restricted bacteria to specific zones, while root dipping allowed extensive xylem colonization due to increased entry points. This extensive colonization permits rapid, passive bacterial propagation without degrading enzymes, as shown with *Burkholderia phytofirmans* strain PsJN in *Vitis vinifera* L. (Compant et al., 2008). These findings highlight the adaptability of these strains in plant colonization. Furthermore, their presence in the stomata and around the trichomes may raise further questions about their impact on plant physiology.

To conclude, this study, utilizing a combination of culture-dependent methods, metabarcoding and confocal analysis, not only underscores the diverse range of seed-transmitted endophytic bacteria but also their dynamic nature and significant potential to influence plant growth under various conditions. It highlights the complexity of plant-microbe in-

teractions and the adaptive capabilities of endophytes within ecological niches.

Looking ahead, key questions about the longevity of these interactions persist, particularly whether older seeds are more susceptible to losing these beneficial partnerships, which could impact germination and seedling vigor. Further research is needed to determine the transmission of these bacterial communities to subsequent generations and assess the long-term stability and evolutionary implications of these interactions. Exploring the influence of these microorganisms on the floral microbiome could provide insights into their effects on plant reproduction and health. Additionally, studying variations across different cultivars could reveal the genetic or environmental factors that influence microbial community composition and functionality. The interplay between endophytes and external microbial populations also warrants investigation, as it could crucially affect the resilience and adaptability of the core microbiota.

From a practical point of view, exploiting specific compositions of the core microbiota to adapt microbial treatments could help sustainable agricultural practices, improving crop resistance, yield and health. Future research should also aim to compare metabarcoding and functional analyses to deepen our understanding of these interactions and their practical applications in agriculture.

CHAPTER VI. Humic substances increase tomato tolerance to osmotic stress while modulating vertically-transmitted endophytic bacterial communities

Salomé Lengrand[1*], Benjamin Dubois[2], Lena Pesenti[1], Frederic Debode[2] and Anne Legrève[1*]

[1] Université catholique de Louvain (UCLouvain), Earth and Life Institute, Louvain-la-Neuve, Belgium

[2] Walloon Agricultural Research Centre (CRA–W), Gembloux, Belgium
[*]Correspondence: Salomé Lengrand (salomelengrand@hotmail.com) and Anne Legrève (anne.legreve@uclouvain)

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1. Abstract

While humic substances (HS) are recognized for their role in enhancing plant growth under abiotic stress by modulating hormonal and redox metabolisms, a key question remains: how do HS influence the microbiota associated with plants? This study hypothesizes that the effects of HS extend beyond plant physiology, impacting the associated bacterial community. To explore this, we investigated the combined and individual impacts of HS and osmotic stress on tomato plant physiology and roots endophytic communities. Tomatoes were grown within a sterile hydroponic system which allowed to focus on seed-transmitted endophytic bacteria. Moreover, sequencing the 16S-ITS-23S region of the *rrn* operon (˜4,500 bp) in a metabarcoding assay using the PNA-chr11 clamp nearly eliminated reads assigned to *Solanum lycopersicum* and allowed species-level identification of these communities. Our findings revealed that HS, osmotic stress and their combined application induce changes in bacterial endophytic communities. Osmotic stress led to reduced plant growth and a decrease in *Bradyrhizobium* sp., while the application of HS under osmotic stress resulted in increased tomato growth, accompanied by an increase in *Frigoribacterium* sp., *Roseateles* sp., and *Hymenobacter* sp., along with a decrease in *Sphingomonas* sp. Finally, HS application under non-stress conditions did not affect plant growth but did alter the microbial community, increasing *Hymenobacter* sp. and decreasing *Sphingomonas* sp. This study enhances understanding of plant-endophyte interactions under stress and HS application, highlighting the significance of the vertically-transmitted core microbiome in tomato roots and suggesting new insights into the mode of action of HS used as biostimulant.

2. Introduction

Climate change imposes various challenges on agriculturally important crops, including extreme weather events, temperature fluctuations, and, most notably, increased drought frequency (Zhou et al., 2024). Tomato (*S. lycopersicum*), the leading horticultural crop, yielded 186.1 million tons in 2022 (FAOSTAT, 2024). Drought, in particular, threatens to reduce tomato yields by up to 40% (Hammond et al., 2022; Kopecká et al., 2023).

To address this constraint, the use of biostimulants—substances or microbial inoculants that improve nutrient efficacy, stress tolerance, and crop quality—has gained attention (du Jardin, 2015). These sustainable products are increasingly important in mitigating the adverse effects of climate change, including the growing severity of droughts (du Jardin,

2015; Rouphael and Colla, 2018). Humic substances (HS), major components of soil organic matter resulting from the decomposition of plants, animal and microbial residues, are one of the main categories of plant biostimulants (du Jardin, 2015). HS are a mixture of supramolecular conformations composed of relatively small and heterogeneous molecules associated by weak bonds (Piccolo, 2002). They benefit plants indirectly by enhancing soil quality and its microbial community, providing carbon, improving soil aeration and stability, and facilitating nutrient uptake (Elkins and Nelson, 2002; Magdoff and Weil, 2004; Nardi et al., 2002; Piccolo, 2002). They also directly influence plant growth by activating hormonal pathways, particularly those of auxin, abscisic acid and ethylene (Canellas et al., 2015). Some studies highlighted that HS contain auxins or have structural similarities with auxin and interact with hormone-cell receptors to initiate hormonal pathways (Canellas et al., 2002; Piccolo, 2002; Trevisan et al., 2010). Such activation leads to the stimulation of proton pumps, improving nutrient import within plants, increasing cell wall loosening and division, and enhancing root system development (Mora et al., 2010, 2012; Morsomme and Boutry, 2000; Muscolo et al., 2013; Nardi et al., 2002; Olaetxea et al., 2019; Quaggiotti, 2004; Trevisan et al., 2010; Zandonadi et al., 2007). The impact of HS on plants also comes from the activation of reactive oxygen species (ROS) pathways and the overexpression of antioxidant enzymes, which prime the plant without causing irreversible damage (Berbara and Garcia, 2014; García et al., 2016; Schiavon et al., 2010). The beneficial effects of HS under water stress have been observed in various plants, including rice—where it helped protect cell membrane permeability—and *Brassica napus*, with reported increases in chlorophyll content, as well as in maize and tomato among others (Eyheraguibel et al., 2008; Galambos et al., 2020; Garcia et al., 2014; Lotfi et al., 2015). Despite in-depth studies on these products, our understanding of their biostimulation mechanisms remains incomplete. Their impacts are probably not only due to a direct influence on the plant but might also involve interactions with intermediary microorganisms (da Silva et al., 2021b).

Plants are known to harbor microbial communities within their tissues, known as endophytes, whose composition is shaped by various biotic and abiotic factors (Lengrand et al., 2024). These plant-microbe interactions are crucial for the adaptation and survival of both plants and microbes under stressful conditions (Meena et al., 2017; Meenakshi et al., 2019; Ullah et al., 2019a). For instance, bacterial endophytes can enhance plant growth through several mechanisms, such as improving nutrient uptake, producing hormones, and generating beneficial metabolites (Malinowski and Belesky, 2000; Ullah et al., 2019a). Endophytes can be acquired from the environment or transmitted through seeds, with each group playing a vital role in plant health. Seed microbiota serve as a reservoir of microbial taxa that have co-evolved with plant hosts, providing functions that support plant survival (Geisen et al.,

2017; Hardoim et al., 2012; Johnston-Monje and Raizada, 2011). Moreover, this vertical transmission ensures the continuity of certain microorganisms from parent plants to their offspring, potentially promoting their beneficial growth. This process contributes to overall plant health and fosters beneficial endosymbiotic relationships (Barret et al., 2016; Cope-Selby et al., 2017; Hardoim et al., 2012; Rudgers et al., 2009; Shade et al., 2017).

Although HS and beneficial bacteria are effective as stand-alone biostimulants, numerous studies have shown that their positive impact on plant growth and stress tolerance is enhanced when used in co-inoculation (Canellas et al., 2013, 2015; dos Santos et al., 2021; da Silva et al., 2021a; Olivares et al., 2015). For instance, Galambos et al. (2020) reported the combined use of HS with *Paraburkholderia phytofirmans* and *Pantoea agglomerans* that significantly promoted tomato growth, suggesting a complementary effect. This finding aligns with research by de Melo et al. (2018) and Olivares et al. (2015), which demonstrated improved plant vitality and stress tolerance when HS were used alongside microbial inoculants. In maize, Canellas et al. (2013) observed that HS application with *Herbaspirillum seropedicae* activated key metabolic pathways, enhancing the plant response to environmental challenges. Similarly, Baldotto et al. (2010) observed that such co-inoculation strategies led to significant improvements in nutrient uptake and overall plant health. The beneficial effects of co-inoculation of HS and PGPB are likely due to multiple factors: (1) increased bacterial colonization and penetration (Nardi et al., 2009; Olivares et al., 2015, 2017; Piccolo, 2002; Canellas et al., 2008; Puglisi et al., 2008; Canellas et al., 2013), (2) activation of plant metabolic pathways that complement those induced by bacteria and HS individually (Galambos et al., 2020; Aguiar et al., 2016) and (3) direct effect of HS on bacterial metabolism (Wang et al., 2024). Since HS are known to influence co-inoculated bacteria, it stands to reason that they would similarly impact the communities of endophytic bacteria within plants.

In the present work, we hypothesize that HS influence plant growth and stress resilience concurrently with modifications in plant-associated bacterial communities and that investigating these effects could help understanding the mechanism of action of these molecules. To distinguish the direct effect of HS on the plant and their indirect effect by influencing the soil microbiome, a hydroponic system in sterile conditions was also developed allowing to focus only on seed-transmitted endophytic bacteria. This study analyzed the effects of HS on tomato growth under both non-stress and osmotic stress conditions, comparing the endophytic communities in roots across these different scenarios. The objectives were to determine (i) the impact of osmotic stress, (ii) the influence of HS, and (iii) the combined effects of HS and osmotic stress on both plant growth and the composition of the bacterial endophytic community.

3. Material and methods

3.1. Impact of HS on plants growth

Experimental design and sampling

The experiment followed a completely randomized design. Four treatments were tested: (1) 'Control'; (2) 'PEG' for osmotic stress; (3) 'HS' for humic substances and (4) 'PEG + HS' for HS application under osmotic stress. Every treatment had six biological replicates and the experiment was repeated three times.

Tomato plants and growth conditions

S. lycopersicum seeds (var. Moneymaker) were disinfected in $< 5\%$ sodium hypochlorite for ten minutes under orbital shaking and washed three times in sterile distilled water for 20 minutes. This concentration and duration of disinfection were selected to prevent any microbial growth on solid media post-disinfection while ensuring a high germination rate (over 95%). Seeds were germinated in a sterile box for ten days, seven days in the dark and three days under light, to maintain optimal humidity for germination. Seedlings were then transferred into a custom-designed sterile hydroponic system which consisted of two Erlens (150 mL) filled with Hoagland's solution adapted for *S. lycopersicum* (NH_4NO_3: 0.04 $g.L^{-1}$; $Ca(NO_3)_2 \cdot 4H_2O$: 0.413 $g.L^{-1}$; KNO_3: 0.2035 $g.L^{-1}$; KH_2PO_4: 0.137 $g.L^{-1}$; $MgSO_4 \cdot 7H_2O$: 0.123 $g.L^{-1}$; $MnSO_4 \cdot 5H_2O$: 0.265 $mg.L^{-1}$; H_3BO_3: 0.7 $mg.L^{-1}$; $CuSO_4 \cdot 5H_2O$: 0.075 $mg.L^{-1}$; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$: 0.004 $mg.L^{-1}$; $ZnSO_4 \cdot 7H_2O$: 0.3 $mg.L^{-1}$; Fe EDDHA: 0.03 $g.L^{-1}$). This system was enclosed in an autoclavable culture bag with a 0.02 µm filter (Sun bag, transparent, B7026-100EA) to allow gas exchange, supported by a plastic frame and a sterile plant potholder, ensuring a controlled environment (Figure VI-1). Tomato plants were cultivated in a growth chamber under a 16h/8h (light/dark) photoperiod regime, 70% relative humidity, and temperature of 24° C/22° C (day/night).

For the osmotic stress treatment ('PEG'), plants were cultivated in Hoagland's solution supplemented with 10% w/v polyethylene glycol 6000 (PEG 6000), chosen based on preliminary tests to quickly induce stress without causing plant death. This adjustment shifted the osmotic potential from -0.02 MPa to -0.2 MPa. Plants treated only with HS ('HS') were grown in hydroponic solution supplemented with humic and fulvic acids at a concentration of 500 $\mu L.L^{-1}$, determined as optimal in

preliminary experiments. A third set ('PEG + HS') received both PEG 6000 and humic and fulvic acids in their hydroponic solution. Control plants were grown in standard Hoagland's solution. Both osmotic stress and HS were introduced one week after transferring the plants to the Erlens. The addition of HS, with or without PEG, did not significantly alter the osmotic potential of the solution (p value > 0.1) (Table S-9). The PEG and biostimulant are added to the hydroponic solution before it is autoclaved, thus preventing any contamination from the PEG or biostimulant. The system was maintained for three weeks, and hydroponic solutions were replenished every week under sterile conditions. This duration was chosen to allow the analysis of rapid effects on plant physiology and morphological responses without compromising plant viability.

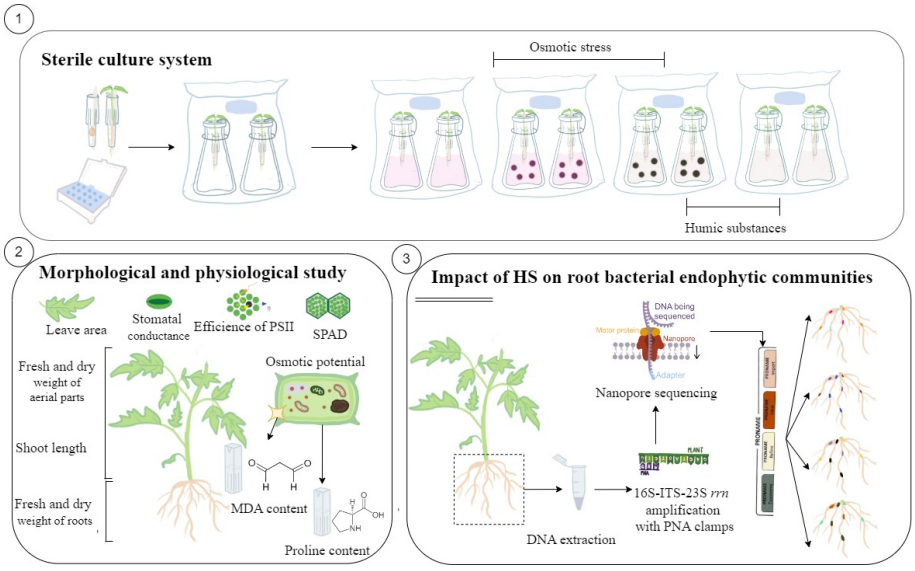

Figure VI-1: Schematic of the experimental workflow.

(1) Culture under sterile condition was made first in a germination system composed of a sterile tips box filled with sterile water and tips in which absorbent paper and seeds were placed. At the two cotyledons stage, the seedlings were transferred into a custom-designed sterile hydroponic system filled with Hoagland's solution adapted for tomato plants enclosed in an autoclavable culture bag with a 0.02 µm filter to allow gas exchange. After one week of adaptation, both osmotic stress and humic substances were applied in the hydroponic solution, plants were grown during three weeks and the sterile hydroponic solution was replaced and treatments applied every week. (2) At the harvest, effects of HS, osmotic stress and both were evaluated through morphological and physiological analysis including shoot length, fresh and dry weight of aerial parts and roots, leave area, stomatal conductance, efficiency of the photosystem II, SPAD, osmotic potential of leaves and proline and MDA contents in leaves.(3) Tomato plants grown under sterile conditions were harvested after three weeks and DNA of roots was extracted before 16S-ITS-23S rrn amplification (4,500 bp) with a new design PNA clamps and Nanopore sequencing. Raw data were analyzed with PRONAME tools.

Humic substance composition

The commercial biostimulant Humifirst® from Tradecorp is a liquid formulation containing 12% humic and 3% fulvic acids (weight/weight solution), extracted from leonardite. Its stable composition ensures reliable experimental outcomes, which is crucial for this type of research. The content of Humifirst and its characteristics inferred from a chromatographic analysis, have been meticulously calibrated and were previously detailed by Tahiri et al. (2016a) (Table VI-1).

Table VI-1: Chemical characteristics of HS extracted from leonardite (Tahiri et al., 2016a,b)
Dissolved organic carbon

Parameter	Unit	Leonardite (HHS)
pH	-	10.2
EC	ms/cm	9
Total N	$mg.L^{-1}$	80
NH_4^+	$mg.L^{-1}$	20
Cl^-	$mg.L^{-1}$	<10
DOC*	$mg.L^{-1}$	18770
Fe	$mg.L^{-1}$	50
Co	$mg.L^{-1}$	0.022
Cr	$mg.L^{-1}$	0.059
Cu	$mg.L^{-1}$	0.091
Ni	$mg.L^{-1}$	0.049
Pb	$mg.L^{-1}$	0.2
Zn	$mg.L^{-1}$	0.114
As	$mg.L^{-1}$	0.0185
Cd	$mg.L^{-1}$	0.0011
Hg	$mg.L^{-1}$	0.00129

Assessment of morphological and physiological parameters of tomato plants

At harvest, morphological and physiological variables were analyzed. The leaf area was scanned and analyzed using ImageJ. The stomatal conductance and the chlorophyll content were measured for each leaf at the four leaves stage using a porometer AP4 and a SPAD chlorophyll meter, respectively. Measurements of stomatal conductance and relative chlorophyll content were taken at the center of each leaf, not on the vein. The efficiency of the photosystem II was evaluated with a fluorimeter with two indicators: Fv/Fm which represents the maximum quantum yield of photosystem II and qPSII which is the actual quantum yield. The osmotic potential of leaves was measured with a Vapor Pressure Osmometer VAPRO®. The proline and malondialdehyde (MDA) contents of leaves were quantified according to the protocol of Bates et al. (1973) and Heath and Packer (1968) respectively. Briefly, both protocols are based on a colorimetric determination measured with a spectrophotometer. The experiment has been reproduced three times. However, the proline and MDA determinations were only performed in one trial.

Statistical analysis

The experiment was repeated three times and data were analyzed with RStudio. After validating data for normal distribution (Shapiro-Wilk test, p value > 0.05) and variance homogeneity, each experiment was analyzed individually, and a two-way analysis of variance (ANOVA) was used to demonstrate non-significant differences between the three trials (p value > 0.05). Data from the three experiments were pooled and significant differences among treatments were assessed with Student's t-test ($p \leq 0.05$). As the stomatal conductance and the relative chlorophyll content were measured on each leaf of plants, the results were analyzed according to a mixed model with the "plant" parameter. Endophytic communities associated with the roots were analyzed only for the third repetition.

3.2. Impact of osmotic stress, HS and combined effects on endophytic communities

Plant growth, sampling and DNA extraction

During the third and final assay of HS application on tomato growth under non-stress and osmotic stress conditions, impact on endophytic

communities was evaluated. At the harvest, five plants per treatment (Control, HS, PEG+HS and PEG) were surface-disinfected. Briefly, entire tomato plants were first placed in NAP buffer (124 mM Na_2HPO_4) and sonicated in an Ultrasonic Cleaning Baths (VWR®) for 1 min to dislodge bacteria on the surface. Plants were then submerged in sodium hypochlorite solution (5.25%) for 6 min and rinsed with sterile water (Ruiz-Perez and Zambrano, 2017). The intact plants were dried on sterile paper. The sterilization was checked by making an imprint of plants on Luria Broth Agar (LBA) and plates were checked for two weeks (Fitzpatrick et al., 2018b). Disinfection was carried out on all plants to avoid the disinfectant solution penetrating the interior of the plants. Only the roots were kept to study the endophytic communities, based on the results of da Silva et al. (2021b) and De Hita et al. (2020). Each root system was ground separately in liquid nitrogen and DNA was extracted from 50 mg using a modified CTAB-based method. Briefly, NaCl (100 µL, 5M) was added before adding isoamyl alcohol: chloroform to reduce high polysaccharides concentration in the extractant (Fang et al., 1992). DNA pellet was diluted in 20 µL of nuclease-free water.

PCR amplification of a ribosomal marker of endophytic bacteria with three Peptides Nucleic Acid (PNA) clamps

Extracted DNA was amplified using the primers 16S-8F (5'-AGRGTTY-GATYMTGGCTCAG-3') and 23S-2490R (5'-CGACATCGAGGTGCC-AAAC-3') targeting the 16S-ITS-23S region of the ribosomal operon (*rrn*) (˜4,500 bp) of bacteria (Karst et al., 2021). The universality of these primers was tested by comparing the genera amplified with three commonly used primer pairs (Lengrand et al., submitted to Phytobiomes Journal). PCR mixture (25 µL total volume) contained 2×GoTaq Long PCR Master Mix, 0.4 µM of each primer, 2 µM of mPNA (to block mitochondrial amplification), 2 µM of pPNA (to block plastid amplification) (Lundberg et al., 2013), 2 µM of PNA-chr11 (to block chromosome 11 amplification of *S. lycopersicum* var. Moneymaker) (Lengrand et al., submitted to Phytobiomes Journal) and 20 ng of DNA. The PCR thermal program consisted of an initial denaturation of 2 min at 95° C followed by 30 cycles of 20 s at 95° C, 60 s at 74° C, 30 s at 55° C, 5 min at 72° C and a final extension 10 min at 72° C. The GoTaq Long PCR Master Mix was chosen for its ability to work in the presence of HS (Matheson et al., 2014), since they are inhibitors of PCR (Sidstedt et al., 2015). For each sample, PCR was performed in three replicates and pooled before purification to assure homogeneity. The amplicons of the 16S-ITS-23S region of the *rrn* operon were cleaned up with AM-Pure XP beads (Beckman Coulter) using a 0.5X beads-to-sample ratio. Quantity and quality were evaluated using a Quantus™ Fluorometer (Promega) and a Nanodrop spectrophotometer (ThermoFisher Scien-

tific), respectively. PCR products were visualized on a 0.8% agarose gel.

Library preparation

Libraries were prepared using the Ligation Sequencing Kit V14 (SQK-LSK114) (Oxford Nanopore Technologies, Oxford, UK) according to the manufacturer instructions. Briefly, amplicons were processed for end repair using the NEBNextUltra II End Repair/dA-tailing Module (New England Biolabs, Ipswich, MA, USA) and sequencing adapters were attached. Libraries were sequenced with a MinION device (Oxford Nanopore Technologies) using Flongle Flow Cells (R10.4.1) during 24h.

Sequencing data processing and analysis of diversity

Raw data were analyzed with PRONAME (Dubois et al., submitted to Frontiers in Bioinformatics). Briefly, raw data were imported into PRONAME, adapters and primers were trimmed, and a graph of simplex and duplex read distribution was constructed. Data were filtered by keeping only duplex reads with a length between 3500 and 5000 bp with a minimum quality score of 15. PRONAME clustered reads with a percentage of identity of 90%, centroid sequences were polished using Medaka and chimera sequences were discarded. The taxonomic analysis of representative sequences was carried out using the blastn standalone tool (v2.15.0) (Camacho et al., 2009) from BLAST command line applications, and the curated rEGEN-B database included in the PRONAME pipeline and dedicated to bacterial 16S-ITS-23S *rrn* operon region. Composition analysis of data was done using the physeq package (McMurdie and Holmes, 2013) in RStudio and results were visualized with ggplot2 package (Wickham, 2014). Alpha diversity was calculated using the vegan R package (Oksanen and Simpson, 2009) with Observed features, Shannon entropy and Pielou eveness followed by a Kruskal-Wallis test. Beta diversity was analyzed by Principal Coordinates Analysis (PCoA), using ape package (Paradis, 2024) and by performing an analysis of similarity (ANOSIM) test using vegan package (Oksanen and Simpson, 2009). Beta diversity was analyzed at the species and genera levels. Finally, identification of taxonomic groups with statistically significant differences in abundance between treatments were analyzed using ANCOM-BC (Lin et al., 2022) on QIIME2 v2024.2 (Bolyen et al., 2019). This method acknowledges sources of variation in microbiome datasets, including unequal sampling fractions and differences in sequencing efficiency, to attempt to limit the effect of group unevenness due to the microbiome variability among individuals in our study. The Operational taxonomic units (OTU) were summarized at the genus level.

4. Results

4.1. Impact of HS on plant growth in non-stress and osmotic stress conditions

In the absence of stress, HS application did not significantly affect most of the measured growth parameters (Figure VI-2, VI-3) A minor reduction in the dry weight of roots was noted in comparison to 'Control' plants, without a noticeable difference in the fresh weight of roots (Figure VI-2B,D). In contrast, a slight but not significant enhancement in the fresh weight of the aerial parts was observed (Figure VI-2A).

Plants grown under polyethylene glycol ('PEG')-induced osmotic stress showed a general decrease in growth. Indeed, a reduced fresh and dry weight, shoot length, and leaf area were observed after three weeks of treatment (Figure VI-2). In parallel, tomato plants showed an accumulation of proline in the aerial parts (Figure VI-3 A) and stomatal closure (Figure VI-3 E) combined with a reduced osmotic potential of leaves (Figure VI-3 C). In parallel with these modifications, plants were subjected to oxidative stress, as evidenced by an increase in lipid membrane peroxidation resulting in elevated MDA content in the aerial parts (Figure VI-3 B). Osmotic stress resulted in no significant alterations in the relative chlorophyll content (Figure VI-3 D), nor in the efficiency of photosystem II (Table S-10).

Under osmotic stress, the addition of HS significantly enhanced aerial parts and root fresh weight (484% and 339%, respectively) as well as aerial parts and root dry weight showing increases of 324% and 239%, respectively (Figure VI-2 A, B, C and D). This increase is observed in parallel with a leaf area enlargement (Figure VI-2, E) and an increase in shoot length (Figure VI-2 F). Moreover, plants treated with HS under osmotic stress showed a reduced MDA content(Figure VI-3 B) and an increased stomatal conductance (Figure VI-3 E) compared to plants under osmotic stress. Finally, both 'PEG' and 'PEG + HS' plants exhibited an increase in proline content and a similar reduction in osmotic potential (Figure VI-3 A and C).

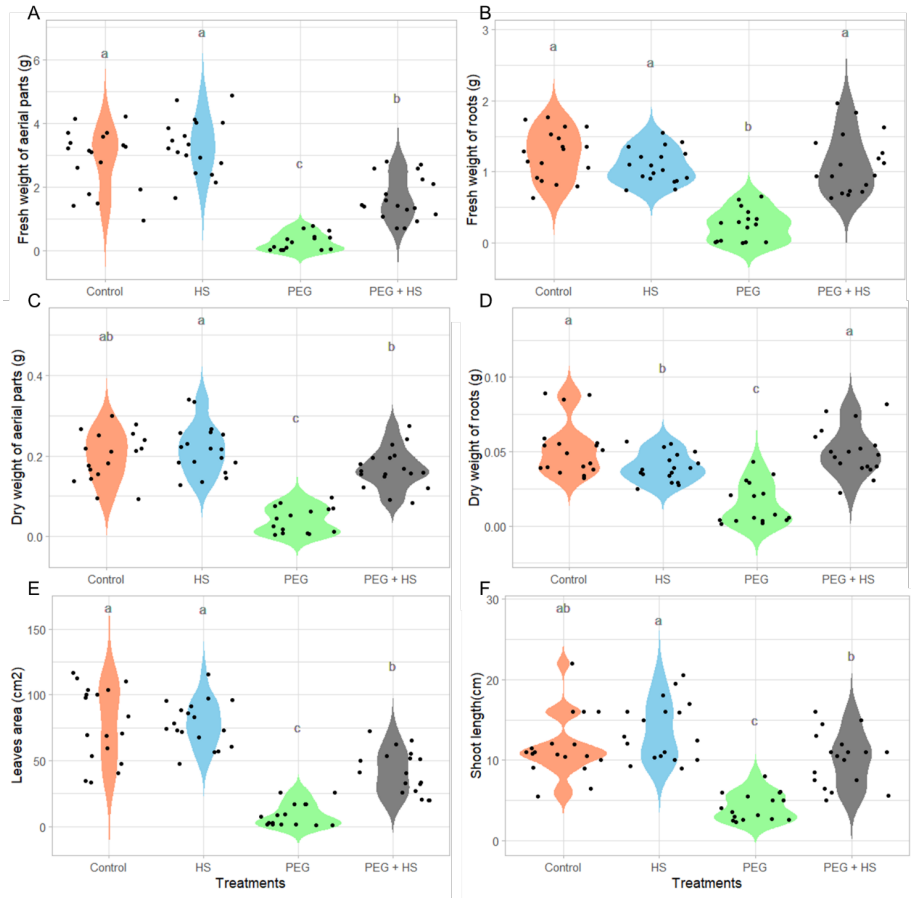

Figure VI-2: Impact of osmotic stress and humic substances on morphological traits of tomato.

Fresh and dry weight of aerial parts (A, C) and roots (B, D), leaf area (E), and shoot length (F) of untreated plants (Control), non-stress condition and treated with humic substances (HS), plants under osmotic stress (PEG), and plants under osmotic stress and treated with HS (PEG + HS) after three weeks of treatment. Multiple comparisons were done with the Wilcoxon test (p value < 0.05). $N = 18$.

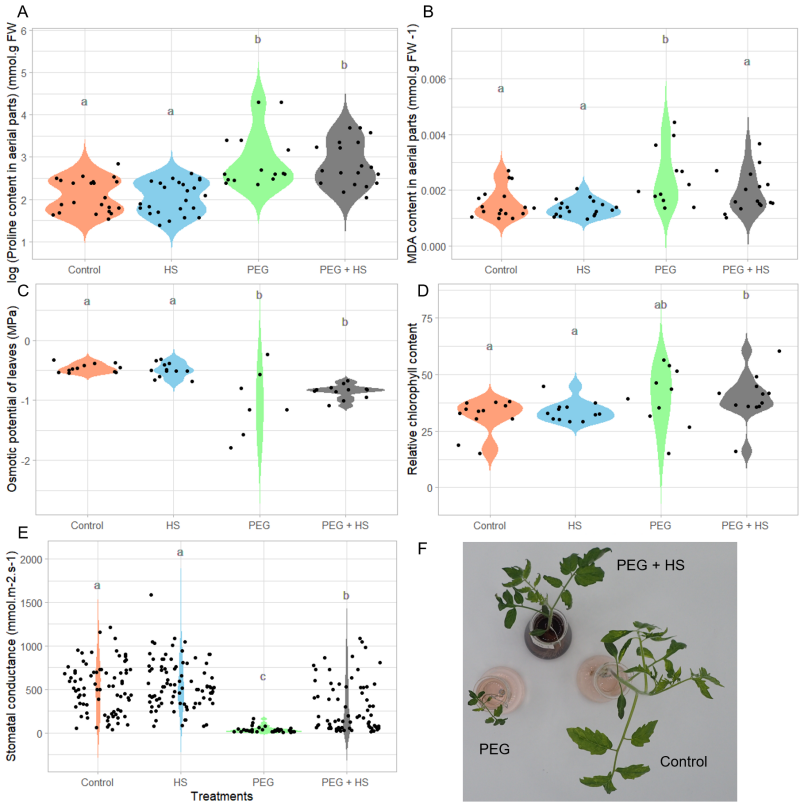

Figure VI-3: Impact of osmotic stress and humic substances on physiological traits of tomato.

Proline and malondialdehyde (MDA) content in aerial parts (A, B), osmotic potential and relative chlorophyll content of leaves (C, D), and leaf stomatal conductance (E) of untreated plants (Control), non-stress condition and treated with humic substances (HS), plants under osmotic stress (PEG), and plants under osmotic stress and treated with HS (PEG + HS) after three weeks of treatment. Images of plants treated with PEG, PEG + HS and control are shown in (F). Multiple comparisons were done with the Wilcoxon test (p value < 0.05). $N = 18$.

4.2. Shift induced by osmotic stress and humic substances in endophytic bacterial community composition

The metabarcoding Nanopore sequencing of 20 samples resulted in 613,942 high-quality duplex reads. One sample from the HS treatment was excluded due to an insufficient number of reads, resulting in four samples for this treatment. The bioinformatic reconstruction of root endophytic communities identified 59 distinct representatives sequences classified into five phyla. For reasons of simplicity, we will call them OTUs. Pro-

teobacteria emerged as the most predominant phylum across all conditions, with relative abundances of 99.84%, 75.33%, 99.33%, and 84.02% in the Control, HS, PEG, and PEG + HS treatments, respectively.

Genera with high relative abundance were similar in the Control and PEG-treated groups, comprising *Bradyrhizobium*, *Methylobacterium*, *Ralstonia*, and *Sphingomonas*. In the HS treatment, *Frigoribacterium* and *Hymenobacter* appeared in high abundance alongside *Bradyrhizobium*, *Methylobacterium*, and *Ralstonia*. Finally, the combined PEG + HS treatment reduced the major genera to *Bradyrhizobium*, *Frigoribacterium*, and *Ralstonia*. *Bradyrhizobium* was predominantly found in all treatments except under PEG conditions, where *Sphingomonas* accounted for nearly half of the observed sequences (Figure VI-4).

Bacterial richness was consistent between Control and HS treated plants. However, following osmotic stress treatment, a significant decrease was observed in bacterial richness both in PEG and PEG + HS treated plants with significant difference in alpha-diversity analysed with Shannon entropy and Pielou eveness (Figure S-8, Figure S-9, Figure S-10).

Distinct patterns emerged in the beta diversity among different treatments, as illustrated by the Principal Coordinates Analysis (PCoA, Figure S-12). Despite some intra-treatment variability observed in the samples (Figure S-11), ANOSIM analysis confirmed significant differences in the beta diversity of the endophytic community across the four treatments at both the genus level (p-value=0.0349, $R^2 = 0.30689$) and species level (p-value=0.0297, $R^2 = 0.29165$). These variations were significantly influenced by the application of humic substances (HS), as detailed in Table VI-2).

Notably, applying the biostimulant without osmotic stress led to an increase in the *Hymenobacter* genus (Bacteroidota phylum) and a reduction in Proteobacteria levels by decreasing the *Sphingomonas* genus (Figure VI-4). This observation was confirmed by the ANCOM-BC results (Figure VI-5). At the species level, there was an enrichment of reads assigned to *Hymenobacter* sp. BRD128 and a reduction of *Sphingomonas aerolata*.

Combining HS and osmotic stress induced an increase in the genera *Frigoribacterium*, *Roseateles*, and *Hymenobacter*, as well as a decrease in the abundance of *Sphingomonas* compared to Control plants (Figure VI-4, VI-5).

Finally, osmotic stress application did not significantly alter the beta diversity (Figure S-12) but did induce a reduction in *Bradyrhizobium* genus abundance (Figure VI-4, VI-5, B).

Table VI-2: Analysis of similarity of the impact of both osmotic stress and/or HS on bacterial endophytic communities in the roots of tomato plants at the genus and species levels.

Treatment comprise both humic substances (HS) and polyethylene glycol (PEG) application.

	GENUS		SPECIES	
	P-value	R^2	P-value	R^2
Treatment	0.0349*	0.30689	0.0315*	0.26163
HS application	0.0084**	0.16202	0.0137*	0.1369
PEG application	0.2420	0.0635	0.1511	0.0749

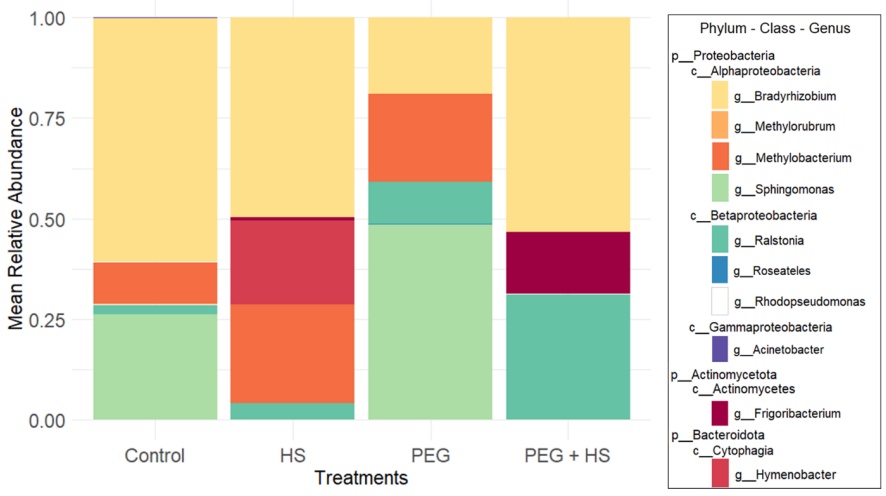

Figure VI-4: Mean relative abundance of endophytic bacterial taxa, at genus level, in the roots of tomato plants according to the treatment applied.
'Control', 'HS', 'PEG' and 'PEG + HS' stand for untreated plants, no stress and treated with humic substances (HS), osmotic stress, and osmotic stress and treated with HS ($n = 6$).

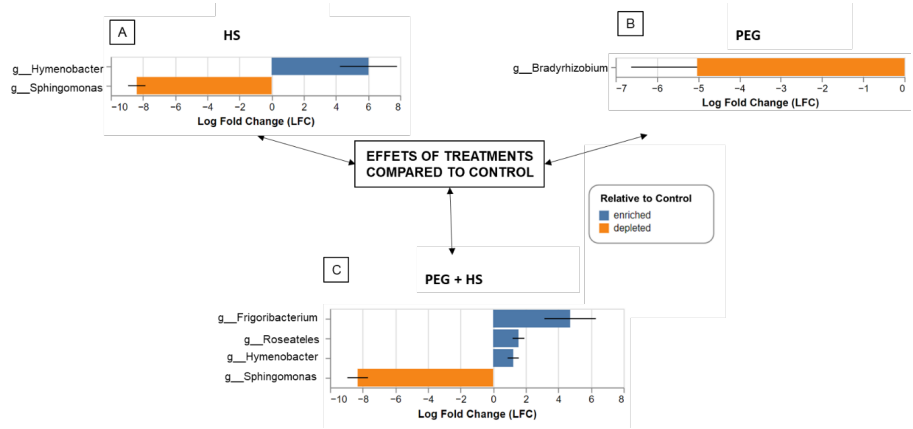

Figure VI-5: Bacterial genera enriched or depleted in tomato roots treated with humic substances (HS) (A), under osmotic stress (B), and under osmotic stress and treated with HS (C), compared to control plants.

Significant differences in relative abundance of taxa at the genus level were inferred using the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) tool.

5. Discussion

This study employed a sterile hydroponic system to isolate the direct effects of HS on plant physiology from interactions with soil microbes. In this particular system, the results indicate that under osmotic stress, HS application enhanced tomato resilience, evidenced by increased shoot and root weights, leaf area, shoot length, total chlorophyll content, and stomatal conductance. This growth enhancement is likely due to HS protective effects, activating antioxidant enzymes and thereby reducing oxidative stress, as indicated by lower MDA content in leaves. The observed decrease in MDA levels post-HS application points to reduced lipid membrane peroxidation, a marker of oxidative damage. This aligns with other studies showing that HS application under stress conditions leads to a healthier plant state through enhanced antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and peroxidase (POX) (Aguiar et al., 2016; García et al., 2012; Lotfi et al., 2015; Matuszak-Slamani et al., 2022). For example, in the experiment of Matuszak-Slamani et al. (2022) on soybeans under osmotic stress in hydroponics, humic acid (HA) application led to an increase in plant length and chlorophyll content in parallel with an upregulation of the antioxidant defense system. Similar results were observed in sugarcane treated with HA under water stress displaying better growth than control plants with increased stomatal conductance,

transpiration, and net photosynthesis. This induced tolerance was also correlated with an activation of antioxidant enzymes activities (Aguiar et al., 2016). On the contrary, HS application did not significantly affect tomato growth under non-stress conditions. This result aligns with existing literature in hydroponics showing that HS-containing biostimulants have limited effects in unstressed environments (Aguiar et al., 2016; García et al., 2012; Lüdtke et al., 2021; Matuszak-Slamani et al., 2022).

The result of this experiment revealed that the increase of tomato resilience to osmotic stress is associated with its direct interaction with plants or its phytobiome and not with an indirect effect through the soil. Since seed-transmitted endophytic communities of plants are now well recognized to play a role in plant health (Pandey et al., 2022; War et al., 2023), we deepened the taxonomic characterization of these communities by PCR amplification and sequencing from total DNA extracted from disinfected roots. The use of the PNA-chr11, tailored specifically to reduce *S. lycopersicum* var. Moneymaker amplification (Lengrand et al., submitted to Phytobiomes Journal), and primers targeting the 16S-ITS-23S *rrn* operon enabled us to achieve species-level identification of endophytes. The primary microbiota present in the roots and transmitted through seeds were identified as *Bradyrhizobium*, *Methylobacterium*, *Ralstonia*, *Frigoribacterium*, *Hymenobacter*, and *Sphingomonas*, with specific reads assigned to *B. sp. SK17*, *M. oryzae*, *M. sp. FF17*, *M. sp. NMS14P*, *R. pickettii*, *F. sp. NBH87*, *H. sp. BRD128*, and *S. aerolata*. This finding aligns with the results of Lengrand et al. (submitted to Phytobiomes Journal) which identified the core microbiome of *S. lycopersicum* var. Moneymaker transmitted from seed to shoots and roots as *R. pickettii*, *S. aerolata*, *B. sp. SK17*, and *P. aquatile*. Additionally, the genera *Frigoribacterium* and *Hymenobacter* were also found in the leaves and roots of plantlets grown in sterile conditions.

The composition of the endophytic microbiome in tomato plants is highly sensitive to environmental conditions. In this study, the tomatoes were grown under sterile conditions, and the hydroponic solution containing PEG, HS, or both was autoclaved before use. Therefore, the observed shifts in the microbiome are not attributable to external microorganisms.

Notably, the osmotic stress alters bacterial community composition by reducing alpha-diversity in both HS-treated and untreated plants. A similar reduction of bacterial richness in roots of *Atractylodes lancea* grown under PEG-induced osmotic stress was observed in the experiment of Wang et al. (2022). Additionally, the application of PEG alone induced a notable shift in the root endophytic community composition, specifically reducing the prevalence of the *Bradyrhizobium* genus.

Under both stress and non-stress conditions, the application of HS led to an increase of Bacteroidetes, especially of *Hymenobacter*, with a reduction in Proteobacteria with an almost complete suppression of *Sphingomonas*. Interestingly, the same conclusion was drawn in the experiment of da Silva et al. (2021b) in rice roots endophytic communities suggesting a consistent effect across different plant species. This influence of HS on bacterial communities seems to extend beyond plant-associated microbiota in the literature as a similar shift after HS application was also observed in wastewaters and lakes (Hutalle-Schmelzer and Grossart, 2009; Luo et al., 2019). Indeed, in ammonium-rich wastewater from landfill leachate, HS application diminished Proteobacteria while enhancing Bacteroidetes with a significant change for aerobic ammonium-oxidizing bacteria (AOB), crucial for nitritation-anammox processes (Luo et al., 2019). Similar adjustments were also observed in the microbiota of oligotrophic Lake Stechlin, where HS addition specifically enriched groups within Alphaproteobacteria, Bacteroidetes, and Deltaproteobacteria, indicating a direct HS effect on microbiota rather than an indirect plant-mediated one (Hutalle-Schmelzer and Grossart, 2009). The interaction between HS and microbial communities may be explained in part by the degradation of HS by bacteria. Under anaerobic conditions, bacteria involved in the anaerobic ammonium oxidation (Anammox) process contribute to HS degradation (Liang et al., 2009). Indeed, reduced HS can act as electron donors for anaerobic organisms utilizing alternative electron acceptors, allowing these microorganisms to derive energy from HS by using carbon sources as acetate (Liang et al., 2009; Lipczynska-Kochany, 2018). This hypothesis might account for the observed shift, particularly for *Hymenobacter*, the predominantly favored genus, which includes numerous species known to function as ammonia-oxidizing bacteria (Garcia-Lopez et al., 2019; Zhang et al., 2020). In addition, HS contain a high concentration of dissolved organic carbon (DOC) (Table VI-1), which can contribute to microbial nutrition (Tahiri et al., 2016a,b). Apart from DOC, the other nutrients in HS should be negligible in plant or microbial nutrition compared with the nutrients present in the hydroponic solution, since they represent, for those present in larger quantities (N and Fe), concentrations a thousand times lower.

In parallel, Actinobacteria have also been found to be active in degrading HS in freshwater and estuarine environments (Haukka et al., 2005; Hutalle-Schmelzer and Grossart, 2009; Rocker et al., 2012; Lipczynska-Kochany, 2018). However, an increase in the Actinobacteria phylum, particularly the *Frigoribacterium* genus, was only observed in plants under osmotic stress that were treated with HS. This increase was notably absent when either the biostimulant or the osmotic stress was applied alone. Interestingly, an increase in Actinobacteria within the root microbiome has already been correlated with enhanced drought resilience in plants (Fitzpatrick et al., 2018a; Martins et al., 2023; Naylor et al.,

2017; Santos-Medellín et al., 2017; Schimel et al., 2007; Tocheva et al., 2016). Xu et al. (2021) demonstrated that an Actinobacteria enrichment in drought stress conditions may be partially due to a reduction of iron homeostasis (increase in insoluble Fe^{3+} in dried soils and reduction of plant Fe transporters) within the root under drought stress. Indeed, the root's reduced iron uptake and increased iron storage lead to low available iron concentrations in the root benefiting bacteria proficient in scavenging the limited iron, to the detriment of less capable ones. This hypothesis gained support from the observation of Xu et al. (2021) where external application of iron interrupts the enrichment of Actinobacteria and in parallel, reduces drought stress tolerance of sorghum. In our experiment, osmotic stress induced in hydroponic systems might not affect Fe^{3+} availability, but HS could create soluble nutrient complexes (with Fe and phosphate among others) absorbable by plants (Spark et al., 1997). However, this absorption mainly involves fulvic acids and not larger humic acids molecules that only interact with cell walls and cannot be absorbed (Nardi et al., 2002). Consequently, the formation of such complexes coupled with diminished water uptake due to osmotic stress, could mimic drought stress effect on reduced Fe availability and increase Actinobacteria relative abundance. The increased abundance of Actinobacteria in link with a higher plant drought tolerance is reported in numerous studies (Ebrahimi-Zarandi et al., 2023). A variety of mechanisms are used by these bacteria, including increasing nutrient availability via phosphate and potassium solubilization, siderophore production, and nitrogen fixation, as well as the production of ACC deaminase, phytohormones, volatile organic compounds, extracellular polysaccharides, and osmolytes (Boukhatem et al., 2022). They also activate the plant's antioxidant system and induce the expression of stress-responsive genes. A meta-transcriptomic analysis conducted by Xu et al. (2018b) on sorghum roots under drought stress revealed significant alterations in Actinobacterial gene expression across different functional categories highlighting the critical roles of carbohydrates, amino acid transport, and ATP-binding cassette (ABC) transporters. *Frigoribacterium* genera, representing the major increase of Actinobacteria in this study, was already known to solubilize phosphate and to produce siderophores with ACC deaminase activity (Boukhatem et al., 2022; Ebrahimi-Zarandi et al., 2023) as exemplified by the strain *F. faeni* 801, isolated from halophytes and with high tolerance to salt stress (Zhou et al., 2017).

Finally, the genus *Roseateles* which abundance was increased under combined osmotic and HS treatment, encompasses species known for their remarkable degradation capabilities. Among these, the strain *R. depolymerans* KCTC42856 stands out for its ability to break down aliphatic polymers in biodegradable plastics (Lee et al., 2016). Similarly, *Ro. chitinivorans* HWN-4^{T} has been identified as a promising candidate for bioremediation, capable of mitigating environmental contaminants (Sisinthy and Gundlapally, 2020). In the experiment of Le

et al. (2023), further insights from antiSMASH analysis highlighted the significant variability in secondary metabolite profiles across *Roseateles* strains, suggesting a rich repertoire of defense mechanisms against competitive microbial species (Le et al., 2023). Considering the characteristics of this genus, it was probably more resilient to osmotic stress induced by PEG and had the ability to surpass competing bacteria by producing certain metabolites to gain access to limited resources.

In summary, endophytic communities carried by tomato seeds and transmitted to the roots were distinctly influenced by osmotic stress and the application of HS, either individually or in combination. The variations following the application of HS can be attributed to several hypotheses: (1) the composition of the HS may favor certain genera capable of using these compounds, indicating a direct nutritional impact, specifically for the *Hymenobacter* genus (Liang et al., 2009; Lipczynska-Kochany, 2018; Luo et al., 2019). (2) Indirectly, HS could modify plant metabolism such as root iron homeostasis and favor specific phyla such as Actinobacteria with the *Frigoribacterium* genus (Fitzpatrick et al., 2018a; Nardi et al., 2002; Xu et al., 2021). (3) The recruitment of certain microbes could respond to HS-induced mild stress, activating plant defense mechanisms (Berbara and Garcia, 2014; da Silva et al., 2021b; García et al., 2016). (4) The effects of HS could also be independent of endophytic composition, leading to a parallel benefit for plants and a change in their microbiota communities (Berbara and Garcia, 2014; da Silva et al., 2021b; García et al., 2016).

In conclusion, this study investigated the combined and individual impacts of HS and osmotic stress on plant physiology and endophytic communities, revealing a possible correlation between endophytic communities and plant physiology, even in sterile environments. This underscores the importance of seed-borne bacteria as primary inoculum. Moreover, HS application under osmotic stress increased tomato growth parameters in concert with the abundance of *Frigoribacterium* sp., *Roseateles* sp., *Hymenobacter* sp. and reduced *Sphingomonas* sp., highlighting the potential of HS to enhance certain plant-endophyte relationships. This opens new perspectives on the mode of action of HS, possibly by interacting with endophytes.

Future research should explore the role of plants in the shifts observed in endophytic bacterial communities following the application of HS and PEG. This could involve creating synthetic microbial communities based on metabarcoding results to directly test the effects of HS and PEG on bacterial strains and communities, as well as using RNAseq to identify the most expressed bacterial genes under different conditions.

Additionally, a promising avenue for future research involves exploring how HS induce shifts in endophytic bacterial communities, guided

by several key hypotheses. One hypothesis suggests that HS may favor bacterial species capable of using HS as electron donors in the anammox process. This could be tested by creating knockout mutants of specific genes involved in this pathway, such as hydrazine synthase (*hzsA*, *hzsB*, *hzsC*) and hydrazine dehydrogenase (*hdh*) (Wang et al., 2016; Kuenen, 2008). Another hypothesis posits that HS form complexes with iron, reducing its availability and potentially increasing the presence of Actinobacteria (Xu et al., 2021). Testing this could involve reducing iron levels in hydroponic solutions instead of applying HS to see if same effects are observed. Moreover, it is also hypothesized that HS might induce mild stress in plants, leading to shifts in bacterial communities. This could be investigated using tomato mutants lacking the *rop9* gene, which regulates ROS formation (Puli et al., 2024). These lines of inquiry could provide valuable insights into the mechanisms behind HS-induced microbial shifts and their impact on plant health and stress tolerance. Finally, studying different tomato cultivars, particularly those with varying stress tolerances and responses to HS, could reveal whether the microbial shifts and beneficial effects of HS are consistent across genetic backgrounds.

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

CHAPTER VII. General discussion

1. Main results

The present work aimed at deepening our knowledge on seed-transmitted endophytic bacteria and at studying the impact of HS on their communities under various stress conditions.

We advanced the characterization of seed-transmitted endophytic bacteria in *S. lycopersicum* var. Moneymaker using a sterile hydroponic culture system. To achieve precise species-level identification of these microbial communities, we designed a new PNA clamp to significantly reduce reads assigned to *S. lycopersicum*, combined with sequencing of the 16S-ITS-23S region of the *rrn* operon (˜4,500 bp). This approach revealed a core microbiota consistently present in seeds, roots, and shoots, which included genera such as *Ralstonia*, *Sphingomonas*, *Bradyrhizobium*, *Roseateles*, *Microbacterium*, and *Stenotrophomonas*, with specific species like *Ralstonia pickettii*, *Sphingomonas aerolata*, and *Paucibacter aquatile*. This core microbiota accounted for 93% of the reads sequenced in shoots and roots, and 99% in seeds (Chapter V).

In parallel, culture-dependent methods under sterile conditions revealed cultivable isolates in seeds, shoots, and roots, with differences from those detected in the metabarcoding study, illustrating the importance of integrating these complementary approaches. Notably, several seed-transmitted endophytic bacteria displayed multiple PGP traits, and specific strains like *Rummeliibacillus stabekisii*, *Peribacillus frigoritolerans*, and *Pseudomonas protegens* enhanced seedling vigor under both osmotic stress and non-stress conditions. These findings underline the pivotal roles of these bacteria in supporting plant growth and enhancing stress resilience, which are discussed in Chapter V.

Through the development of mCherry-tagged mutants for selected strains, we visually tracked bacterial colonization patterns across plant tissues using quantitative PCR, confocal microscopy, and colony counting. This provided critical insights into the dynamics of bacterial colonization and their interaction with the host plant, contributing to our understanding of their roles in plant health and resilience (Chapter V).

Building on these findings, a subsequent study investigated how HS influence the plant-associated endophytic communities under varying stress conditions. Utilizing the same sterile hydroponic system, we were able to isolate the effects of seed-transmitted endophytic bacteria, thereby providing a first view of their interactions with HS and environmental stress. We observed that HS and osmotic stress uniquely modified the bacterial endophytic communities within the roots. Specifically, osmotic stress reduced plant growth and decreased *Bradyrhizobium* sp. abundance, whereas HS application increased *Hymenobacter* sp. and decreased *Sphingomonas* sp. When combined with osmotic stress, HS treatment not only improved tomato growth but also induced shifts in microbial dynamics, increasing *Frigoribacterium* sp., *Roseateles* sp., and *Hymenobacter* sp., while reducing *Sphingomonas* sp (Chapter VI).

These studies collectively enhance our understanding of the complex dynamics between plant-endophyte communities and their response to environmental stresses and biostimulant application. They highlight the potential role of the vertically transmitted core microbiome in maintaining plant health and suggest that HS may modulate these relationships, potentially by favoring beneficial endophytic genera. This research opens new avenues for exploring biostimulant mechanisms and their potential to improve plant resilience through microbiome modulation, underscoring the holistic view of plants as holobionts within biostimulant research. This systemic perspective reshapes our understanding of the phytobiome, enriching our knowledge of plant responses to biostimulants and natural phenomena such as drought stress.

2. Particular methodological advances in our approach

First, we provide a more precise characterization of seed-transmitted endophytic bacteria under sterile conditions by utilizing long-fragment sequencing and developing a new PNA clamp to reduce plant sequence bias. This methodological advancement allows for a more accurate identification of endophytic bacteria, overcoming limitations associated with short-read sequencing and plant DNA contamination. Additionally we developed a bioinformatic pipeline capable of conducting species-level

metabarcoding approach (PRONAME).

Second, we developed mutants of seed-transmitted endophytic bacteria to investigate their initial colonization of seeds and their transmission across plant generations. These mutants have provided valuable insights into the dynamics of endophytic colonization and inheritance, enhancing our understanding of how beneficial microbial communities are maintained and propagated in plants. This understanding is crucial for developing strategies aimed at enhancing plant resilience and productivity through microbiome management. However, it's important to acknowledge the limitations encountered during this research, such as our inability to transform *Bacillus* species. This difficulty could be due to the robust cell wall structure of *Bacillus*, which presents significant barriers to genetic material uptake, or possibly the lack of efficient transformation protocols that are tailored to the unique physiological properties of these bacteria.

3. Plant phenotype is defined by its genome, the environment, and its microbiota

The traditional understanding that plant phenotypes are primarily shaped by genotype-environment interactions (GEI) (Schlichting, 1986) has evolved. Today, plants are recognized as holobionts—cohesive units of host and microbial entities that function synergistically to influence plant health, development, and resilience to environmental stresses (Hassani et al., 2018). This shift necessitates a redefinition of plant phenotypes to include not only genetic and environmental inputs but also the complex interactions within the microbiota they harbor, making the phenotype a dynamic outcome of these interconnected factors: genome, environment, and microbiota. The recognition of plants as holobionts underscores the importance of considering the broader ecological context in which they exist, aligning with the "One Health" approach by highlighting how plant health, influenced by intricate genome-environment-microbiota interactions, is integral to the overall health of ecosystems, animals, and humans (Hoffmann et al., 2022).

Particularly crucial to phenotypic expression are endophytic bacteria that reside within plant tissues, significantly impacting plant health through hormone production, nutrient solubilization, and stress resilience (Lengrand et al., 2024; Afzal et al., 2019; Kandel et al., 2017; Pandey et al., 2022). The close relationship between some endophytes and plants raises intriguing questions about their evolutionary trajectory, including whether they could eventually become integrated as plant organelles, such as mitochondria and chloroplasts. Additionally, the concept of the

seed-endophytome as a matriarchal heritage adds another layer of complexity, suggesting that plants may pass down beneficial microbiota from generation to generation (Truyens et al., 2015).

The phytobiome, acting as an extension of the plant genotype, provides a second layer of protection against pathogens and buffers the plant from extreme environmental conditions (Carrion et al., 2019; Timm et al., 2018). The concept of core microbiota has been proposed to describe the microbial community that is systematically associated with a given plant genotype (Lundberg et al., 2012). Subsequently, the concept of "functional core microbiota" shifts focus from merely identifying microbial species to understanding their essential roles. The functional core microbiota suggests that the most vital microorganisms are those that carry specific genes essential for the holobiont's fitness, regardless of their species. Moreover, it highlights how plants and microbial communities have co-evolved, with plants selecting microbes that not only survive in their environment but actively contribute to their host's adaptation and performance. This selection transcends traditional taxonomic classifications, focusing instead on functional capabilities that bolster the plant's overall health and resilience (Lemanceau et al., 2017).

Further expanding this model, the concept of the hologenome integrates the genetic information of both the plant and its microbial partners. This collective genomic entity enhances the plant's adaptability to varying environmental conditions and its resistance to pathogens, crucial for the survival of sessile organisms (Müller et al., 2016).

4. New vision in biostimulant studies

Our findings have demonstrated that the core microbiome of *S. lycopersicum* remains mainly identical across different plant parts under non-stress conditions (Chapter V). However, under osmotic stress, certain genera become more dominant, likely due to their tolerance or the disappearance of less resistant genera, while the overall composition remains similar. This stability underscores the robustness of the plant-microbiome relationship under normal conditions.

Interestingly, the application of HS under stress conditions caused significant shifts in the microbial community. Previously low-abundance genera became dominant, indicating a real impact of the biostimulant (Chapter VI). This shift suggests that HS may alter nutrient availability or induce stress responses that change the microbial community structure. This result highlights the need for a broader perspective when studying biostimulants. The study of endophytic bacteria alone is not sufficient to fully understand the mode of action of biostimulants but

represents a critical first step in viewing plants as holobionts. In conclusion, incorporating the microbial dimension into biostimulant research is essential.

Recognizing the pivotal role of the plant microbiome and embracing the concept of the plant as a holobiont, we can enhance our comprehension of biostimulant effects. This can be achieved by adopting a multi-omic approach, encompassing metagenomics, transcriptomics, proteomics, and other relevant methodologies (Sarhan et al., 2019). By integrating these diverse techniques, we can gain profound insights into the intricate functional dynamics governing plant-microbe interactions. This holistic strategy capitalizes on a range of advanced methodologies, as outlined in Table VII-1.

Additionally, while our research has primarily focused on bacterial endophytes, it is crucial to expand the scope to include fungal communities. Fungi play significant roles in plant health and stress resilience, yet they are often underrepresented in current studies, partly due to limitations in existing databases.

Overall, integrating these advanced techniques and broader environmental conditions will pave the way for developing more effective biostimulant strategies and sustainable agricultural practices. This holistic approach will not only improve plant resilience and productivity but also contribute to a deeper understanding of the complex interplay between plants and their microbiota.

5. Challenges in endophytic community identification

Identifying endophytic bacteria presents several challenges. Media isolation tends to favor well-studied and cultivable genera, providing a limited view of the microbial community. This method, however, allows for the possession of strains and opens up experimental possibilities. Conversely, amplification and sequencing techniques introduce biases based on what is amplified, potentially overlooking less abundant organisms.

Extracting bacteria within plant cells without degrading their DNA sufficiently to amplify long fragments is challenging. This difficulty is partly due to the differences between Gram-positive and Gram-negative bacteria, which have different sensitivities (de Bruin and Birnboim, 2016). The selection of DNA extraction methods for bacterial analysis depends on the bacterial type. For Gram-positive bacteria, beadbeating combined with chemical lysis (mostly with lysozyme) is most

Table VII-1: Multiples approach to unravel the complexities of plant-associated microbiomes and their interactions with the host plant.

Techniques	Advantages	References
Metagenomics	Provides detailed understanding of microbial community composition and functional potential.	Fadiji and Babalola (2020)
Metatranscriptomics	Elucidates active members and functional phenotypes of microbiomes under various environmental conditions.	Turner et al. (2013)
Metaproteomics	Identifies proteins expressed by microbial communities, giving insights into their functional roles.	Zhang and Figeys (2019)
Metabolomics	Identifies and quantifies small molecules involved in plant-microbe interactions, providing a holistic view of the biochemical landscape.	Pang et al. (2021)
Amplicon sequencing (e.g., 16S rRNA)	Offers insights into the taxonomic diversity of microbiomes across different plant compartments.	Regalado et al. (2020)
Whole genome shotgun sequencing	Reveals the genomic potential and adaptive traits of microbial communities.	Bulgarelli et al. (2015); Xu et al. (2018a)
Culturomics	Creates extensive microbial culture collections for experimental validation and functional analyses.	Sarhan et al. (2019)
Synthetic biology	Engineers microbial genes and constructs synthetic regulatory circuits to improve plant performance.	Ke et al. (2021)
Synthetic microbial communities (SynComs)	Tailored to enhance plant growth and resilience, providing a clear view of microbe interactions with plants.	Vorholt et al. (2017)

effective due to their thick cell walls, while Gram-negative bacteria can be efficiently lysed using chemical or enzymatic methods (Salazar and Asenjo, 2007). DNA of Gram-positive bacteria is more difficult to extract, and some genera such as *Bacillus* produce DNase, which could impact DNA fragmentation during extraction, complicating large amplifications (Kamino and Gulden, 2021). However, in the study of endophytic communities, customizing protocols for individual bacteria isn't feasible because the focus is on the collective community, rather than specific species. Additionally, the presence of the plant introduces complexities that need to be addressed within the protocol. This challenge underscores the absence of comprehensive guidelines for the study of endophytes including protocols for: surface-disinfection, complete extraction, amplification, and sequencing of bacterial endophytes (Compant et al., 2021). Additionally, the existing databases are constrained by historical high sequencing expenses. While the decreasing costs of sequencing are broadening our understanding, the inclusion of new genomes remains primarily reliant on cultivable bacteria or shotgun sequencing. However, the latter is rarely employed for endophytes due to the significant proportion of reads linked to the host plant, thereby hindering thorough analysis of endophytic communities.

6. Perspectives

During this study, we encountered certain difficulties and developed new techniques to overcome them, opening up numerous new research perspectives in this field of study.

6.1. Creation of guidelines for bacterial endophytic community studies

We faced several challenges and uncertainties while developing the protocol to study the endophytic bacterial communities in tomatoes. As explained above, there is a lack of clear guidelines on optimal surface disinfection methods, appropriate extraction techniques, and the potential effects that different extraction and sequencing approaches may have on the observed communities. To answer these questions, it might be useful to create a mock community of bacterial species representative of the tomato endophytome based on species identified as part of the tomato endophytome, both by metabarcoding and by a culture-dependent method. Inoculating plants through seed coating or root drenching with this mock community would enable testing of various extraction methods, potentially leading to the development of effective guidelines for extracting, amplifying, and sequencing endophytic bac-

teria. Concurrently, inoculating plants with mCherry-tagged bacterial mutants could confirm the presence of the introduced bacteria within plant tissues. Their abundance could then be quantified using qPCR, supported by TN7 amplification, in combination with metabarcoding analysis. Validating a protocol that would allow the observation of what has been introduced would be of great assistance for future studies.

6.2. Investigating shifts in bacterial communities under various conditions

Investigating plant roles in microbial shifts following HS and PEG application

One perspective involves examining the role of the plant in the shifts observed in endophytic bacterial communities following HS and PEG application. This research is also based on the creation of a mock community representative of the tomato endophytome in order to test the direct effects of HS and PEG on individual bacterial strains and communities. This first step would give us a clue to the modes of action behind the effect of HS on endophytic communities. Furthermore, performing RNAseq on bacterial strains submitted to osmotic stress and HS would help identify which genes are most expressed under different conditions, providing insights into the pathways induced in bacterial endophytes by HS and PEG application.

Testing hypotheses to explain HS-induced changes in bacterial communities

Exploring how HS induces shifts in endophytic bacterial communities involves several hypotheses. One hypothesis suggests that HS may favor certain bacterial species capable of using HS as electron donors in the anammox process. To test this, creating knockout mutants of genes implicated in the anammox pathway, such as hydrazine synthase (*hzsA*, *hzsB*, *hzsC*) and hydrazine dehydrogenase (*hdh*), would be necessary (Wang et al., 2016; Kuenen, 2008).

Another hypothesis posits the change in bacterial communities after HS application was based on the fact that HS create complexes with iron, leading to a reduction of iron availability for the plants and their microbiota. This reduction should lead to an increase in Actinobacteria, as explained in Chapter VI, which could be responsible for better tolerance to osmotic stress (Xu et al., 2021). One approach to test this hypothesis could be to reduce the iron content in the hydroponic solu-

tion rather than adding the biostimulant, to determine whether the same increase in Actinobacteria occurs and whether the enhanced tolerance to osmotic stress also emerges in parallel.

Additionally, HS application might induce a mild stress in the plant, causing shifts in bacterial communities. Using tomato mutants lacking the *rop9* gene, which regulates the formation of ROS, could help test this hypothesis (Puli et al., 2024).

Investigating the genotype-dependent and field-specific effects of HS on endophytic communities and plant stress tolerance

In order to further elucidate the beneficial effects of HS, an intriguing avenue for future research is to extend the study to a variety of tomato cultivars, particularly those that are either tolerant or sensitive to abiotic stress, to determine whether the shifts in endophytic communities observed in this study are consistent across various genetic backgrounds. In our manuscript, we observed a significant shift in bacterial communities when HS were applied under osmotic stress, and one hypothesis is that this shift could explain the beneficial effects of HS. By comparing several tomato cultivars, including stress-tolerant and stress-sensitive, we could explore several possibilities regarding these interactions. The shift in endophytic communities might be the same across both cultivars, but the beneficial effect of HS may not be observed in both. This scenario would suggest that other cultivar-specific factors play a role in the response of plants to HS application. Second, if the shift in bacterial communities and the beneficial effects of HS are both consistent across cultivars, this would support the hypothesis that the microbial shift is a key mechanism behind the observed benefits. Conversely, if the shifts differ between cultivars, it would suggest a more complex interaction where the plant genotype influences the microbiome response to HS, and therefore, the beneficial effects might be cultivar-specific. This experiment could reveal whether certain cultivars inherently possess microbiomes that are more responsive to HS, thereby enhancing their resilience to stress. Moreover, understanding these genotype-microbiome interactions could aid in the development of targeted biostimulant applications tailored to specific cultivars (Berendsen et al., 2012).

Additionally, it is essential to investigate whether the results observed under controlled hydroponic and sterile conditions can be replicated in field conditions. In the field, plants are exposed to a more complex and diverse microbial community. This raises the question: are the specific endophytic communities influenced by HS in sterile conditions also important in the field, or do other bacterial communities take advantage

and shift due to HS application? Field studies would help determine whether the beneficial microbial shifts observed in controlled conditions are applicable in more variable and challenging real-world environments. It is possible that different bacterial communities, which are more competitive in soil environments, may respond differently to HS, potentially leading to different outcomes in plant growth and stress tolerance.

7. Potential of biostimulant in political and agriculture context

The potential of biostimulants in sustainable agriculture is significantly influenced by evolving political landscapes, particularly in Europe, where changes in regulations are fostering the development and adoption of these products. The European Union's legislative framework, especially the EU Fertilizing Products Regulation (EU FPR), has been pivotal. This regulation not only standardizes the definition and marketing across member states but also emphasizes the EU's commitment to enhancing plant resilience and reducing chemical inputs, which is vital for sustainable agricultural practices.

Biostimulants are poised to play a crucial role in achieving the Sustainable Development Goals (SDGs), particularly those related to responsible consumption and production, and climate action (Kavvada et al., 2022). These products help reduce the environmental footprint of agriculture by minimizing nutrient runoff, enhancing soil health, and improving water and nutrient use efficiency (Halpern et al., 2015). The EU's alignment with these goals through its Green Deal and Farm to Fork Strategy, which aims to reduce the use of chemical pesticides and fertilizers, further underscores the importance of biostimulants in sustainable agriculture (Commission, 2020).

However, the homologation process for biostimulants presents both opportunities and challenges. Compared to biocontrol products, which are regulated as pesticides and require extensive safety and efficacy testing, biostimulants often enjoy a more streamlined and less costly registration process due to their classification under less restrictive regulatory categories. This can lead to faster market entry, making biostimulants an attractive option for companies looking to invest in sustainable agricultural solutions (Pujari, 2023).

Yet, this regulatory ease also brings complications. The simpler pathway for biostimulants can result in a market flooded with products that have poorly understood modes of action and inconsistent efficacy. This situation is compounded by the fact that some products with biocontrol

capabilities might be marketed as biostimulants to avoid the stringent pesticide regulations, leading to further confusion and potential misuse. In parallel, products that have both biostimulant and biopesticide functions need to be registered under different regulatory frameworks (Pujari, 2023). Addressing these issues requires enhanced research collaboration to better understand the modes of action, adaptive regulatory frameworks that can evolve with emerging scientific data, and robust pre-market and post-market monitoring to ensure product efficacy and safety.

Furthermore, transparency is essential to improve stakeholder understanding of biostimulant products, their appropriate uses, and limitations. This approach not only supports the integrity of agricultural inputs but also ensures that they deliver their intended benefits without unintended consequences, thereby fostering sustainable agricultural practices effectively.

In conclusion, while biostimulants hold significant promise for advancing sustainable agriculture, especially within the regulatory environment of the EU, the challenges associated with their regulation and the current gaps in understanding their function must be addressed. This will ensure that the growth of the biostimulant market contributes positively to sustainable agriculture, benefiting the environment, farmers, and societies at large.

8. Endophytes as microbial seed coating

Seed coating is the practice of covering seeds with external materials to improve handling, protection, and, to a lesser extent, germination enhancement and plant establishment (Pedrini et al., 2017). The use of endophytes as seed coatings offers several advantages and disadvantages.

One of the primary advantages of seed coating is that it provides a precise and cost-effective method to deliver microbial inoculants as it reduces the volume of application compared to other methods of delivering microbial inoculants. This method positions microbial inocula in the soil where they are well-placed to colonize seedling roots and protect against soil-borne diseases and pests, making it suitable for large-scale applications (O'Callaghan, 2016). Additionally, seed coating can improve the germination index and seedling establishment in small and tiny seeds, reducing seed wastage (Rocha et al., 2019; Ma, 2019). It also enhances the physical properties of the seeds, facilitating easier planting and achieving uniform seedling emergence (Ma, 2019). Furthermore, seed coatings can protect seeds from being eaten by insects and animals that live in the soil (Heydari et al., 2010).

The environmental benefits of seed coating with microorganisms are significant. It promotes sustainable agriculture by improving plant growth and reducing the need for agrochemical inputs (Rouphael et al., 2017). Coating seeds with beneficial bacterial endophytes helps maintain seed viability and vigor by providing a protective barrier against unfavorable environmental conditions and pathogens, thereby supporting plants during their most vulnerable stages. Endophytes can help plants cope with abiotic stressors, enhance the plant's antioxidant defense system against pathogens, and increase tolerance to biotic (pathogens and pests) and abiotic stress (salt, drought, heavy metals) (Rouphael et al., 2017).

However, there are also disadvantages to using microbial seed coatings. Improper formulation can negatively impact germination rates and seedling growth, highlighting the need for precise formulation and application methods. The development and application of microbial seed coatings can be complex and costly, involving the selection of suitable microorganisms, carriers, and binders (Paravar et al., 2023; Ma, 2019; Rocha et al., 2019). Ensuring the survival and efficacy of microorganisms in the coating over time requires careful consideration of factors such as shelf life and environmental conditions. The interval between covering the seeds and planting them in the field should be minimized, and storage conditions must be controlled to maintain low storability (Bashan et al., 2014; Ma, 2019; Swaminathan et al., 2016). Additionally, the coating process must ensure proper dispersion of microorganisms among the plants (Lally et al., 2017).

Biological seed treatments are likely to be used alongside conventional farming practices, materials, and agrichemicals. It is crucial to test their efficacy under real farm conditions and assess compatibility with commonly used agrichemicals, which can adversely affect beneficial soil microorganisms, particularly impacting rhizobia inoculants (Fox et al., 2007; O'Callaghan, 2016).

In conclusion, while applying endophytes as seed coatings provides considerable advantages for plant growth, protection, and sustainable agriculture, it also introduces challenges that need to be addressed to maximize its effectiveness.

Our study demonstrated that coating seeds with beneficial endophytic bacteria such as *Peribacillus frigoritolerans*, *Rummeliibacillus stabekisii* and *Pseudomonas protegens* facilitated systemic dispersion as seen with TN7 mutants of these bacteria within the plant and enhanced tomato growth under both normal and stress conditions. However, further research is needed to determine whether these bacterial strains can successfully colonize plants in more complex field environments.

9. Endophytes in a global system

Endophytes, traditionally recognized for their vital roles in plant health, extend their significance far beyond mere plant protection and growth enhancement. These symbiotic microorganisms have profound implications for animals and humans (Martínez-Romero et al., 2021). When herbivores consume plants, they ingest these endophytes. Some of them may be digested or not liberated from plant tissues, but others may remain active in the gut, contributing to the digestive health and nutritional efficiency of the host (Thursby and Juge, 2017). This can explain why the guts of herbivores have larger diversity compared to carnivores (Ley et al., 2008). For instance, different species of *Lactobacillus* and *Clostridium* found in herbivores possess enzymes capable of breaking down complex plant polymers like cellulose, thus enhancing digestive efficiency and nutrient absorption (El Kaoutari et al., 2013). This microbial symbiosis not only aids in digestion but also produces beneficial metabolites like short-chain fatty acids, which are crucial for gut health (Ríos-Covián et al., 2016). They can also contribute to the synthesis of essential amino acids or vitamins, fix nitrogen, or provide defense against pathogens.

Moreover, the evolutionary relationship between plants, endophytes, and herbivores underscores the ecological importance of these microbes. Plants provide a habitat for endophytes, which in turn benefit herbivores by enhancing their ability to digest plant materials and by defending against pathogens. This tripartite interaction is evident in the specialized diets of certain animals, such as koalas (Barker et al., 2013) and pandas (Jin et al., 2020), whose gut microbiota are adapted to their unique diets of eucalyptus and bamboo, respectively.

While both endophytic fungi and bacteria are important components of the plant microbiome and play crucial roles in plant health and ecology, the transfer of endophytic bacteria from plants to herbivores/omnivores tends to be more extensive and frequent (Martínez-Romero et al., 2021). Insects among animals seem to have had unique associations with fungi for around 420 million years (de Leon A. et al., 2018). These associations ranged from pathogenic to obligate mutualisms. Some endophytes produce secondary metabolites that can deter herbivorous insects or reduce their growth and reproductive success. Conversely, the ingestion of beneficial endophytes can positively influence the health and fitness of insect herbivores by contributing to the digestive processes by producing enzymes that break down complex plant polymers, thus enhancing nutrient availability (Gibson and Hunter, 2010; Boucias et al., 2012). Moreover, the presence of endophytes in the gut microbiota of insects can enhance their immune responses, helping them fend off pathogens and improving their overall

survival and fitness. Specialized insect endosymbionts in abdominal bacteriomes could have derived from gut bacteria, which in turn derived from endophytes found in plants that may not exist today (de Leon A. et al., 2018).

The human gut microbiota also depends on diet, indeed, transient microbiota may derive from food. There is a direct link between the gut microbiome in humans and the number of vegetables consumed (David et al., 2014; McDonald et al., 2018). The broader implications of these findings extend to human health. For example, leveraging endophytic microbes as probiotics could enhance human and animal health by improving digestion and nutrient absorption. For example, *Lactobacillus plantarum*, a plant endophyte, is used as a probiotic to better resist viral infections (Panigrahi et al., 2017; Endo et al., 2010).

This holistic understanding of endophytes as crucial players in the health of plants, animals, and humans underscores the need for further research and development in utilizing these microorganisms for sustainable agricultural and human health.

CHAPTER VIII. Conclusion

Endophytic bacteria play a pivotal role in plant health and development (Martínez-Romero et al., 2021). These organisms, residing within plant tissues, can notably enhance plant growth, nutrient uptake, and stress resilience through the production of phytohormones, nutrient solubilization, and biocontrol against pathogens (Rosenblueth and Martínez-Romero, 2006).

This thesis specifically examined seed-transmitted endophytic bacteria in *S. lycopersicum*, exploring their role in sustaining plant health and resilience. These bacteria, passed from one generation to the next, could form a stable and beneficial microbial community that supports plant development from its earliest stages (Truyens et al., 2015). In our study, we identified the core microbiota of *S. lycopersicum* using metabarcoding, which includes the genera *Ralstonia*, *Sphingomonas*, *Bradyrhizobium*, *Roseateles*, *Microbacterium*, and *Stenotrophomonas*. Concurrently, we assessed the ability of these bacteria to enhance tomato osmotic stress tolerance through traditional culturing methods. Additionally, by using *mCherry*-tagged mutants of specific strains, we were able to trace the colonization patterns of these endophytes across different plant tissues.

Given the significant role of endophytic bacteria in plant health, it is crucial to consider these communities when studying the effects of biostimulants like HS (da Silva et al., 2021b). Our research demonstrated that both osmotic stress and the application of HS induce changes in the bacterial composition of tomato roots. Specifically, HS application under osmotic stress conditions resulted in enhanced plant tolerance to stress, which was associated with a notable rise in the abundance of the genera *Frigoribacterium*, *Roseateles*, and *Hymenobacter*. These findings highlight the possible synergistic relationship between HS, endophytic bacteria, and plant resilience, suggesting that HS not only affect the plant directly but also modulate the plant-associated microbial commu-

nities, leading to improved plant health and stress resilience.

Our research contributes significantly to the knowledge on interactions within phytobiome by providing a detailed characterization of seed-transmitted endophytic bacteria in tomatoes and highlighting the microbial shift in bacterial communities in the context of biostimulant application.

In conclusion, our research confirms the interest of incorporating the microbial dimension into biostimulant research. By acknowledging the plant microbiome role and considering the plant as a holobiont (Hassani et al., 2018), we can develop a more comprehensive understanding of biostimulant effects. This holistic approach is crucial for developing more effective biostimulant strategies, enhancing plant resilience and productivity in diverse environmental conditions, and contributing to sustainable agricultural practices. Future research should continue to explore the interactions between plants, endophytes, and biostimulants, expanding the focus to include both bacterial and fungal communities to fully understand their roles in plant health and productivity.

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APPENDIX

1. Supplementary tables

Table S-1: Sequences of primers used in this study and sequences of primers used to evaluate their generality

Oligo name	**Forward sequence 5' to 3'**	**Reverse sequence 5' to 3'**	**Amplicon**	**References**
16S-8F	AGRGTTYGATYMTGGCTCAG	CGACATCGAGGTGCCAAAC	*rrn*	Karst et al. (2021)
27F-2241	AGRGTTYGATYHTGGCTCAG	ACCRCCCCAGTHAAACT	*rrn*	Zeng et al. (2013)
A519F-U2428R	CAGCMGCCGCGGTAA	CCRAMCTGTCTCACGACG	*rrn*	Martijn et al. (2019)
27F-534R	AGRGTTYGATYMTGGCTCAG	ATTACCGCGGCTGCTGG	V1-V3	Walker et al. (2015)

Table S-2: NCBI number of bacterial strain used to create PNA-chr11 clamps

NCBI number	NCBI number
MZ435324.1	OP070057.1
OP185364.1	ON908989.1
OM838396.1	MG996857.1
MW928752.1	OP209984.1
MH715221.1	OP185243.1
OP175935.1	ON076056.1
MZ443724.1	ON786554.1
OM585519.1	OP012837.1
ON254198.1	ON063213.1
OM866237.1	OP210298.1
NR_171393.1	OP210312.1
OP080982.1	OP068017.1
OP210275.1	KP768795.1
OP068015.1	OP012824.1
MZ486441.1	OP104215.1
ON398991.1	OP093991.1
JN196541.1	MT853112.1
OP080780.1	ON545981.1
MT827143.1	AF255603.1
ON799333.1	OP080988.1
NR_029287.1	MW722927.1
OP080933.1	MH094649.1
MW310689.1	OP080835.1
OM763958.1	OP210259.1
OP080828.1	OP132369.1
OP168187.1	OP216732.1
OP132304.1	OP209785.1
NR_175468.1	OP210276.1
MW243042.1	OP168512.1
ON014504.1	

Table S-3: Characteristics of primers used for MLSA

Target gene	**Primer name**	**Sequence 5' to 3'**	**Amplicon size (bp)**	**Annealing temperature (°C)**	**Reference**
DNA-directed RNA polymerase subunit beta (*rpoB*)	LAPS5	TGGCCGAGAACCAGTTCCGCGT	1230	50	Nikolić et al. (2018)
	LAPS27	CGGCTTCGTCCAGCTTGTTCAG			
RNA polymerase sigma factor (*rpoD*)	PsEG30F	ATYGAAATCGCCAARCG	743	55	Mulet et al. (2010)
	PsEG790R	CGGTTGATKTCCTTGA			
DNA gyrase subunit B (*gyrB*)	gyrB_aF64_Mi	CTTTCCCTACACGACGCTCTTCCGATCT	340-364	55	Barret et al. (2015)
	gyrB_a353_Mi	GGAGTTCAGACGTGTGCTCTTCCGATCT			

Table S-4: Primers sequences and thermal cycling conditions used to quantify Tn*7*-mutants and *tubulin* gene of *S. lycopersicum*

Target	Forward - Reverse	Amplicon	Thermal cycling conditions	References
Tn*7*	F_Tn*7*_25511: (5'- TCACCAGCTCACCGTCTTTC-3')	66 bp	95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.	This study
	R_Tn*7*_2576: (5'- TTCTTGCCCGCCTGATGAAT-3')			
TUB	TUB-F: (5'- AACCTCCATTCAGGAGATGTTT-3')	180 bp	95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.	Lovdal and Lillo (2009)
	TUB-R: (5'- TCTGCTGTAGCATCCTGGTATT-3')			

Table S-5: Number of reads assigned to bacteria and plant genomes in samples amplified with universal PNA clamps (mPNA and pPNA)

Nbre of reads / sample	**Reads assigned to bacteria**	**Reads assigned to *S. lycopersicum***
Sample1 34,245	19,653 (57.4%)	14,592 (42.6%)
Sample2 32,070	12,652 (39.5%)	19,418 (60.5%)
Sample3 34,953	5,637 (16.1%)	29,316 (83.9%)
Sample4 46,166	20,957 (45.4%)	25,209 (54.6%)
Total 147,434	58,629 (39.8%)	88,535 (60.2%)

Table S-6: Sequences of *S. lycopersicum* genome co-amplified during 16S-ITS-23S *rrn* amplification of bacterial genomes with universal PNA clamps

Identification	**% of identification**	**Accession number on NCBI**	**% of tomato reads**
Solanum lycopersicum genome assembly, chromosome: 11	100%	OU640354.1	91%
Solanum lycopersicum chloroplast, complete genome	100%	OQ473549.1	9%

Table S-7: Effects of seed coating with bacterial strains on germination, roots length and shoot length of tomato under non-stress and osmotic stress conditions.

The significance of the data is assessed by comparing bacterial treatment with control using a Student's t-test, where * $0.05 > p\text{-}value > 0.01$*;* ** $0.01 > p\text{-}value > 0.001$*;* *** $p\text{-}value < 0.001$

Treatments	**% germination**	**% germination**	**Roots length**	**Roots length**	**Shoots length**	**Shoots length**
	0 MPa	**-0.2 MPa**	**0 MPa**	**-0.2 MPa**	**0 MPa**	**-0.2 MPa**
C1 47.2	1	1	7,606***	7,389	6,217	5,672
C1 5B	0,972	0,944	7,156	8,056***	6,445*	6,144***
C1 M38	0,917	0,944	4,083***	5,912	5,889	5,241
C1 M39	0,889	0,944	6,617	6,45	6,322	5,911
C1 M8	1	0,972	7,45*	5,917	5,956	5,417
C2 12	0,972	1	7,256*	7,467***	6,539*	5,928*
C2 20	0,917	0,972	5,956	5,406	6,344	5,834
C2 30B	0,944	0,972	7,2944	6,85	6,317	5,334
C2 52B	0,972	0,972	7,722***	7,428**	6,417	5,817
Control	0,972	0,889	6,089	6,025	5,889	4,25

Table S-8: Bacterial quantification by colony-counting assay in non-disinfected and disinfected plants

Bacterial strains	**Non-disinfected plants**			**Disinfected plants**		
	Roots	**Shoot**	**Leaves**	**Roots**	**Shoot**	**Leaves**
C1M8 (CFU/ g of fresh weight)	5.7×10^8	1.11×10^8	1.71×10^{10}	1.47×10^7	2.82×10^5	1.65×10^7
C1M39 (CFU/ g of fresh weight)	1.19×10^{10}	4.46×10^9	2.13×10^8	6.38×10^5	5.64×10^4	1.64×10^6

Table S-9: Comparison of the osmotic potential of hydroponic solutions of the four treatments

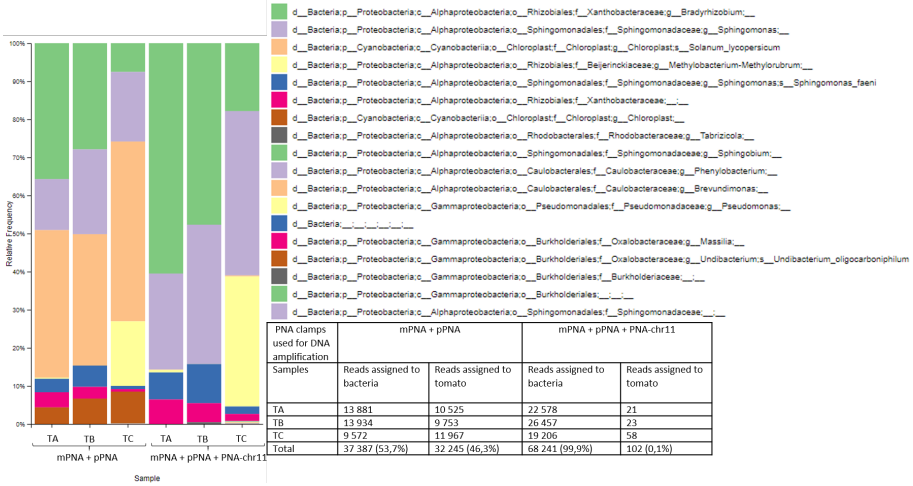
Comparison treatments	**Sum Sq**	**Df**	**F value**	**Pr** $(> F))$
Control vs HS	4.429e-06	1	0.25	0.6433
PEG vs PEG + HS	0.0003662	1	3.4543	0.1124

Table S-10: Comparison of the efficiency of the photosystem II of tomato under non stress "Control" and osmotic stress "PEG" conditions.

Data from the three experiments were pooled and significant differences among treatments were assessed with Student's t-test ($p \leq 0.05$).

Parameters	**Estimate**	**P value**	**Significance**
Fv/Fm	0.009972	0.8617	-
PSII	-0.01147	0.9918	-

2. Supplementary figures



PNA clamps used for DNA amplification	mPNA + pPNA		mPNA + pPNA + PNA-chr11	
Samples	Reads assigned to bacteria	Reads assigned to tomato	Reads assigned to bacteria	Reads assigned to tomato
TA	13 881	10 525	22 578	21
TB	13 934	9 753	26 457	23
TC	9 572	11 967	19 206	58
Total	37 387 (53,7%)	32 245 (46,3%)	68 241 (99,9%)	102 (0,1%)

Figure S-1: Results from Illumina sequencing of the V1-V3 region of the *rrn* operon, specifically amplified from full operon amplicons generated in an earlier step.

This graph displays the relative abundance of reads assigned to Solanum lycopersicum and various endophytic bacterial taxa at the genus level within tomato roots. 'TA', 'TB', and 'TC' represent plant DNA samples amplified with universal PNA clamps (mPNA + pPNA) and with universal primers alongside the new PNA-chr11 (mPNA + pPNA + PNA-chr11).

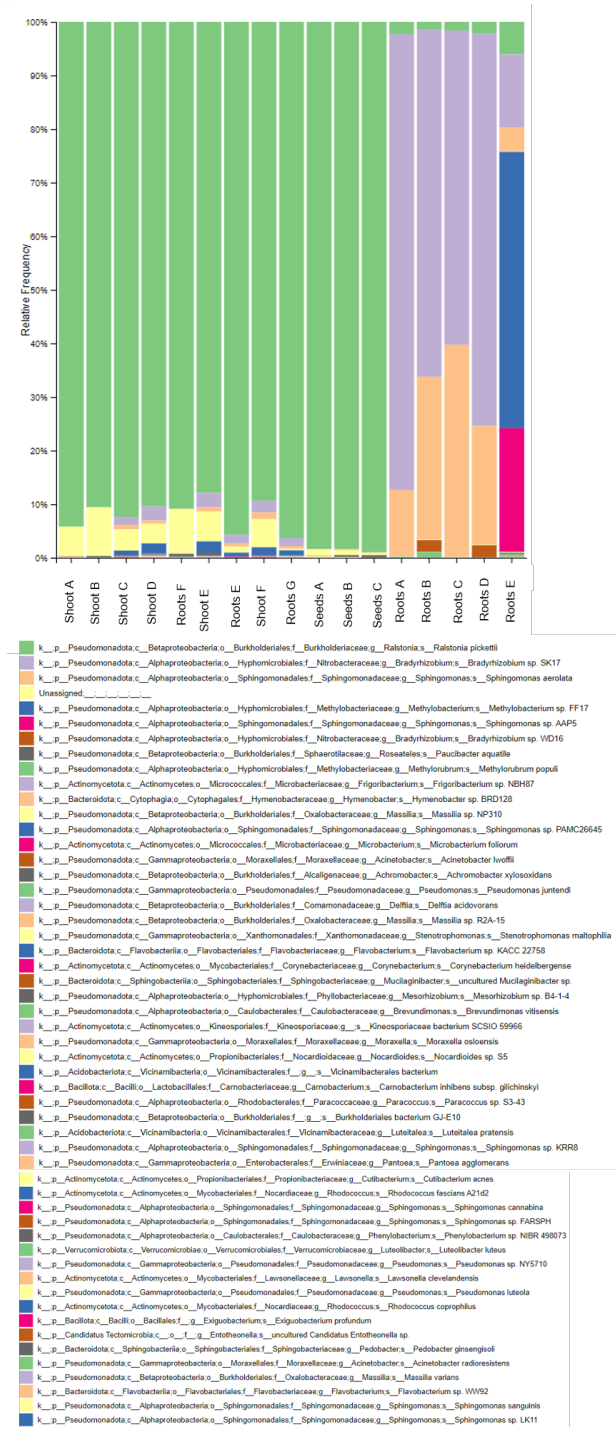
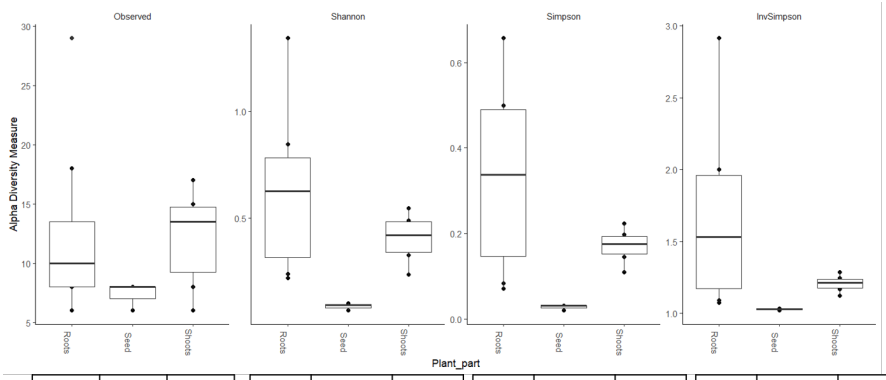


Figure S-2: Results of the Nanopore sequencing of the 16S-ITS-23S *rrn* operon of bacterial endophytic communities.

Seeds (Seeds A, B, C), roots (Roots F, E, G), shoots (Shoots D, E, F), surface-disinfected roots (Roots A, B, C, D, E) and surface-disinfected shoots (Shoots A, B, C).



P-value: 0.2421	Roots	Seed
Seed	0.28	-
Shoots	0.79	0.28

Pvalue: 0.0209	Roots	Seed
Seed	0,036	-
Shoots	0.345	0.036

Pvalue: 0.0209	Roots	Seed
Seed	0.036	-
Shoots	0.345	0.036

Pvalue: 0.0209	Roots	Seed
Seed	0,036	-
Shoots	0.345	0.036

Figure S-3: Alpha diversity indexes between bacterial endophytic communities of *S. lycopersicum* in tomato seeds, roots and shoots of tomato plants grown in sterile conditions for three weeks.
Pairwise comparisons were evaluated using Wilcoxon rank sum exact test.

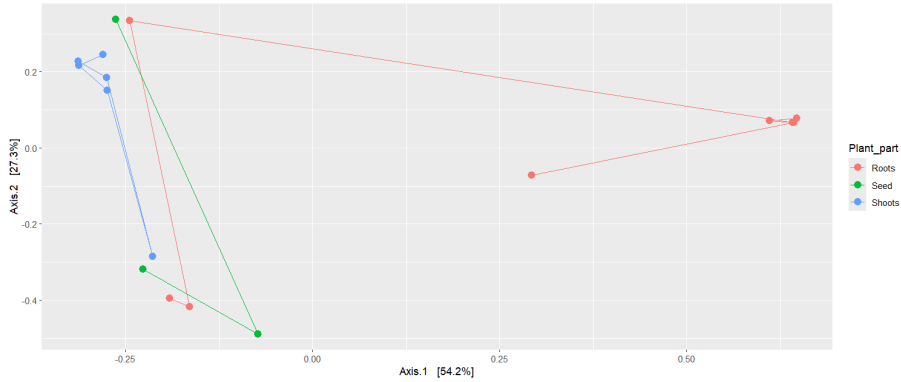


Figure S-4: PCoA plot with sample distances inferred from bacterial endophytic community composition in tomato seeds, roots and shoots of tomato plants grown in sterile conditions for three weeks.
Samples are colored according to the three tested microhabitats. P value = 0.0011, calculated with PERMANOVA.

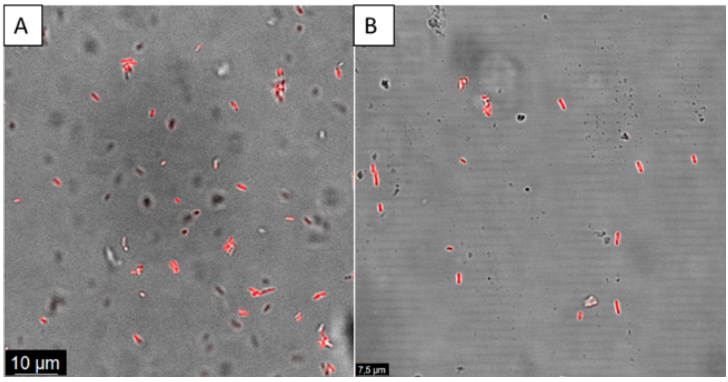


Figure S-5: Free-living bacterial strains tagged mCherry C1M39 (A) and C1M8 (B) observed with a confocal microscope

Analyses of qPCR data					
C1M8 disinfected		p value	C1M8 No disinfected		p value
Leaves	Roots	0,893	Leaves	Roots	0,456
Leaves	Shoots	0,319	Leaves	Shoots	0,535
Roots	Shoots	0,076	Roots	Shoots	0,287
C1M39 disinfected		p value	C1M39 No disinfected		p value
Leaves	Roots	0,152	Leaves	Roots	0,056
Leaves	Shoots	0,158	Leaves	Shoots	0,148
Roots	Shoots	0,153	Roots	Shoots	0,556
Analyses of colony counting data					
C1M8 disinfected		p value	C1M8 No disinfected		p value
Leaves	Roots	0,917	Leaves	Roots	0,658
Leaves	Shoots	0,245	Leaves	Shoots	0,658
Roots	Shoots	0,762	Roots	Shoots	0,447
C1M39 disinfected		p value	C1M39 No disinfected		p value
Leaves	Roots	0,524	Leaves	Roots	0,819
Leaves	Shoots	0,312	Leaves	Shoots	0,819
Roots	Shoots	0,327	Roots	Shoots	0,819

Figure S-6: Analysis of bacterial colonization of C1M8::*mCherry* and C1M39::*mCherry* in plant parts according to the surface-disinfection status.

The bacterial colonization was inferred from qPCR results and from media colony counting. Statistical analysis was performed using Student's t-test ($(p \leq 0.05)$) on Rstudio 4.2.0.

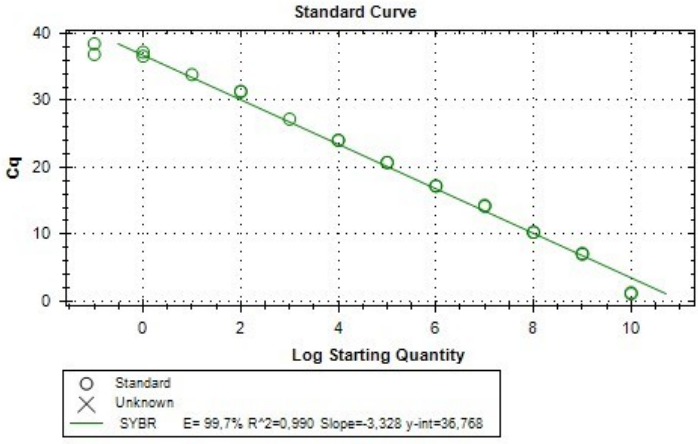
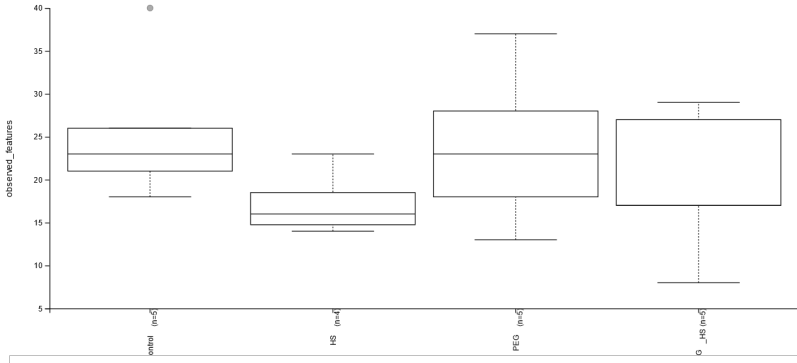


Figure S-7: Efficiency of the primers used in the Tn*7* qPCR and the line equation of the standard curve.



Kruskal-Wallis (all groups)

	Result
H	3.3787798408488143
p-value	0.33682760383840155

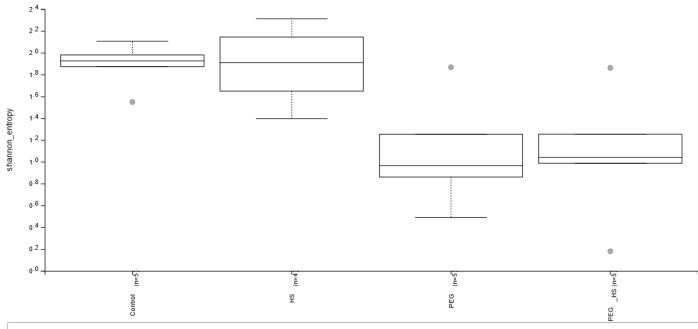
Kruskal-Wallis (pairwise)

Download CSV

		H	p-value	q-value
Group 1	Group 2			
Control (n=5)	HS (n=4)	3.403361	0.065064	0.390382
	PEG (n=5)	0.099387	0.752567	0.752567
	PEG_HS (n=5)	0.889024	0.345742	0.556013
HS (n=4)	PEG (n=5)	1.225210	0.268341	0.556013
	PEG_HS (n=5)	0.558621	0.454817	0.556013
PEG (n=5)	PEG_HS (n=5)	0.537805	0.463344	0.556013

Figure S-8: Alpha diversity (Observed features) between bacterial endophytic communities of *S. lycopersicum* in tomato roots according to the treatment applied.

Comparison between tomato roots treated with humic substances (HS), under osmotic stress (PEG), under osmotic stress and treated with HS and control plants (PEG-HS).



Kruskal-Wallis (all groups)

	Result
H	10.193684210526328
p-value	0.016989504405801603

Kruskal-Wallis (pairwise)

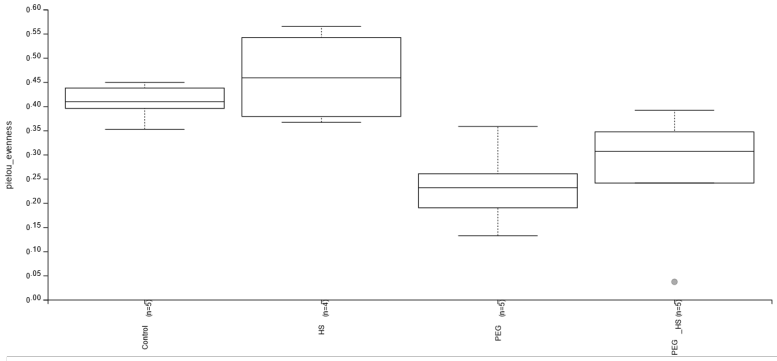
Download CSV

Group 1	Group 2	H	p-value	q-value
Control (n=5)	HS (n=4)	0.000000	1.000000	1.000000
	PEG (n=5)	5.770909	0.016294	0.048881
	PEG_HS (n=5)	5.770909	0.016294	0.048881
HS (n=4)	PEG (n=5)	3.840000	0.050044	0.075065
	PEG_HS (n=5)	3.840000	0.050044	0.075065
PEG (n=5)	PEG_HS (n=5)	0.098182	0.754023	0.904827

Braplot.pptx - PowerPoint

Figure S-9: Alpha diversity (Shannon entropy) between bacterial endophytic communities of *S. lycopersicum* in tomato roots according to the treatment applied.

Comparison between tomato roots treated with humic substances (HS), under osmotic stress (PEG), under osmotic stress and treated with HS and control plants (PEG-HS).(C) Pielou eveness.



Kruskal-Wallis (all groups)

	Result
H	11.507368421052632
p-value	0.009276122597187896

Kruskal-Wallis (pairwise)

Download CSV

		H	p-value	q-value
Group 1	Group 2			
Control (n=5)	HS (n=4)	0.240000	0.624206	0.624206
	PEG (n=5)	5.770909	0.016294	0.032587
	PEG_HS (n=5)	5.770909	0.016294	0.032587
HS (n=4)	PEG (n=5)	6.000000	0.014306	0.032587
	PEG_HS (n=5)	3.840000	0.050044	0.075065
PEG (n=5)	PEG_HS (n=5)	0.534545	0.464702	0.557643

Figure S-10: Alpha diversity (Pielou eveness) between bacterial endophytic communities of *S. lycopersicum* in tomato roots according to the treatment applied.

Comparison between tomato roots treated with humic substances (HS), under osmotic stress (PEG), under osmotic stress and treated with HS and control plants (PEG-HS).

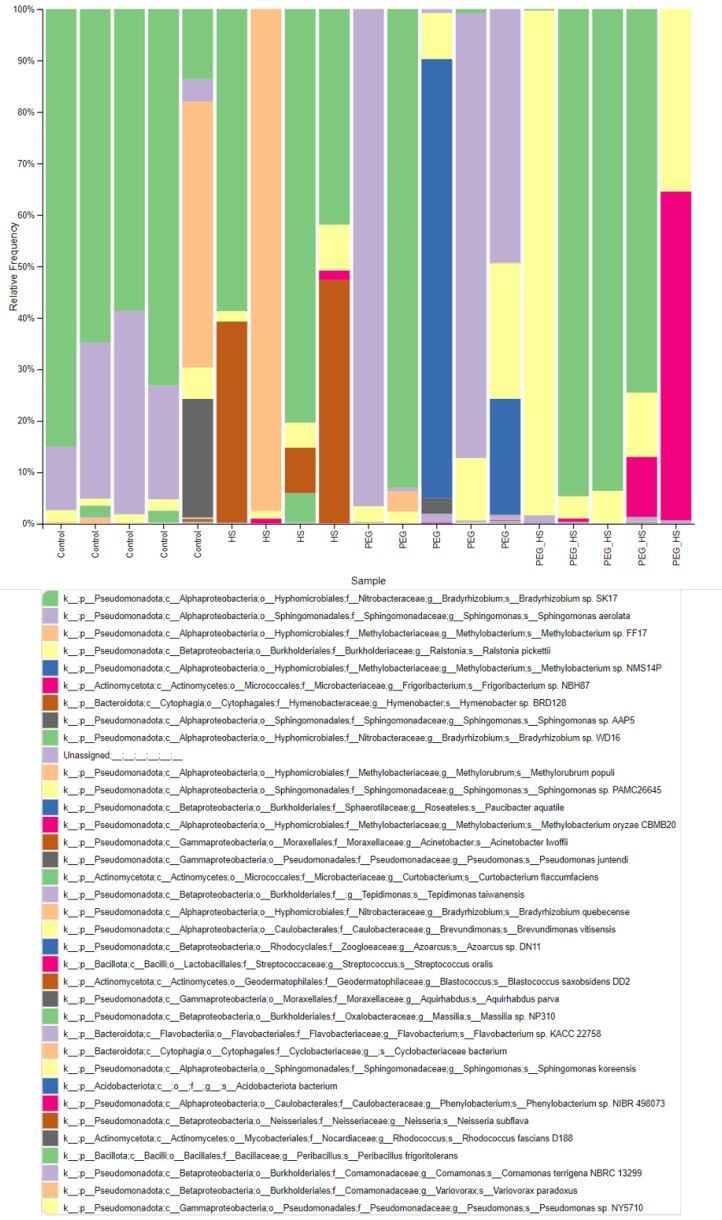


Figure S-11: Relative abundance of endophytic bacterial taxa in the roots of tomato at genus level in each sample.

Control, HS, PEG-HS and PEG stand for plants under non stress, non stress and treated with humic substances (HS), osmotic stress and treated with HS and osmotic stress alone.

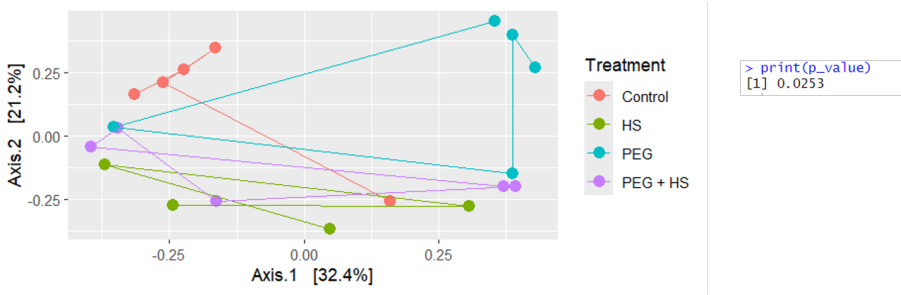


Figure S-12: PCoA plot with sample distances inferred from bacterial endophytic community composition in roots of plants according to the treatment applied.

Plants treated with humic substances (HS), under osmotic stress (PEG), under osmotic stress and treated with HS and control plants (PEG-HS).