Hidden allies:

Seed microbiome diversity and its potential in early plant development and stress resilience



Sasiwimon Siricharoen

Propositions

1. The most effective microbial inoculants already exist in wild plant populations. (This thesis)
2. Plant stress resilience begins with the seed microbiome. (This thesis)
3. Plant science relies too heavily on controlled experiments.
4. Plant research has moved beyond its dependence on model species.
5. With age, good sleep becomes more valuable than good food.
6. The greatest challenge of studying abroad is adapting socially, not academically.

Propositions belonging to the thesis, entitled

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Hidden allies:
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Chapter 1

General introduction

Seeds represent a critical stage in the plant life cycle, supporting reproduction, dispersal, genetic diversity, adaptation to changing environments, and ensuring species reproduction and survival (Baroux & Grossniklaus, 2019; Beckman et al., 2020; Fernández-Pascual et al., 2019). For example, the seed quality is a major determinant of successful germination, seedling establishment, and crop productivity (Finch-Savage & Bassel, 2016; Yang & Wen, 2017). High-quality seeds promote uniform emergence, efficient use of water and nutrients, and improved resilience to biotic and abiotic stresses, ultimately contributing to higher productivity (Finch-Savage & Bassel, 2016; Reed et al., 2022; Weissmann et al., 2023). For decades, seed quality has been considered to be primarily determined by plant genotype and environmental conditions (He et al., 2014). However, recent studies have shown that seeds harbour diverse microbial communities, known as the seed microbiome, which can also influence seed performance and quality (Liu et al., 2024b; Shade et al., 2017). Seed maturation is a critical stage during which key seed quality traits are established, and the seed microbiome is assembled in the seeds (Ahmed et al., 2025; Brunel-Muguet et al., 2025; Matilla, 2025). These seed-associated microorganisms can be transmitted across plant generations, contributing to seed quality and early plant growth (Xu et al., 2026). One important aspect of the seed microbiome is the production of chemical compounds, including volatile organic compounds (VOCs), which can positively influence seed germination, seedling development, and plant stress resilience through plant-microbe interactions (Tyc et al., 2020). When germinating in the soil (the seed's natural environment), seeds are exposed to different environmental conditions and changes (seasons), and therefore, seed germination and seedling establishment are highly sensitive to abiotic stresses such as salinity and drought (Long et al., 2023; Uçarlı, 2020). Understanding the roles of seed-associated microorganisms in early plant growth and stress resilience, as well as how the seed microbiome is assembled, is becoming increasingly important for improving crop adaptation to climate change and supporting sustainable agriculture.

Early plant development: Seed germination and seedling establishment

Seed germination and seedling establishment represent critical phases in the plant life cycle that determine plant growth and crop productivity (Finch-Savage & Bassel, 2016). Their success is strongly influenced by seed quality traits, including viability, vigour, dormancy, and longevity, which collectively influence the ability of seeds to germinate under favourable conditions, establish rapidly and uniformly, and maintain performance during storage (Obura & Lamo, 2024; Wimalasekera, 2015). In this context, dormancy is a state that delays germination during adverse environmental conditions, enabling seeds to survive until

conditions become favourable for seedling establishment (Bentsink & Koornneef, 2008). Germination capacity reflects the ability of seeds to germinate successfully, while seed vigour refers to the potential for rapid and uniform emergence and the production of healthy seedlings, particularly under environmental stress conditions (Reed et al., 2022). These seed quality traits are largely established during seed maturation through complex physiological, biochemical, and hormonal changes (Artur et al., 2026). During this stage, seeds gradually lose water and acquire desiccation tolerance, while protective molecules (e.g., LEA proteins, sugars, antioxidants and stress granules) protect cells from dehydration damage, enabling long-term survival and viability during storage (Leprince et al., 2017; Smolikova et al., 2020). Seeds also accumulate storage reserves (e.g., starch, proteins, and lipids) to support later germination and seedling growth (Baud et al., 2008), while hormonal regulation, particularly through abscisic acid (ABA) and gibberellic acid (GA), controls dormancy establishment and germination in response to environmental conditions (Ali et al., 2022; Nguyen et al., 2025b). Collectively, these processes establish key seed quality traits that determine seed survival, germination performance, and seedling establishment (Finch-Savage & Bassel, 2016).

Germination represents a critical phase in the plant life cycle, marking the transition from a dry dormant seed to radicle emergence through water uptake and metabolic activation (e.g., utilization of stored nutrients and protein synthesis) (Bewley, 1997; Yildirim et al., 2025). The germination process can be divided into three phases. In the first phase, a dry, metabolically inactive seed undergoes rapid water uptake during imbibition. The second phase involves metabolic activation, including reserve mobilization and protein synthesis, which progressively weakens the seed coat (testa) prior to radicle protrusion. In the third phase, radicle protrusion exhibits the completion of germination (Bewley, 1997; Makhaye et al., 2021). The germinated seed then develops into a seedling, during which the radicle forms the primary root and the shoot emerges, establishing independent growth (Nelson & Larson, 1984).

Seed germination is regulated by external environmental cues, such as water availability, temperature, and oxygen, together with endogenous factors, including hormones and genetic background (Koornneef et al., 2002; Yan & Chen, 2020; Zhao et al., 2024). Environmental signals are perceived and translated into internal responses that regulate hormonal interactions and gene expression during germination, while genetic background, including variation among plant genotypes, influences seed sensitivity to these signals. Together, these factors contribute to differences in germination performance, seed vigour, dormancy behaviour, and stress tolerance under different environmental conditions (Barua

et al., 2012; Xu et al., 2024). These regulatory processes involve extensive transcriptional reprogramming of genes associated with hormonal signalling, metabolic activation, reserve mobilization, cell wall modification, and embryo development during the transition from dormancy to germination and early seedling growth (Liew et al., 2024; Narsai et al., 2017; Sajeev et al., 2024).

Early developmental stages are highly sensitive to abiotic stresses such as drought and salinity (de Medeiros et al., 2023). During germination, these stresses restrict water uptake during imbibition, impair metabolic reactivation (e.g., respiration and protein synthesis), disrupt reserve mobilization of starch, lipids, and proteins, and alter hormonal balance through reduced GA and increased ABA signalling, thereby delaying or inhibiting radicle protrusion (Ali & Elozeiri, 2017; Borges et al., 2026; Ibrahim, 2016; Uçarlı, 2020). In response, seeds activate stress-responsive gene expression associated with ABA signalling, osmotic adjustment, antioxidant defence, and ROS homeostasis, thus enhancing stress tolerance and regulating germination under adverse environmental conditions (Luo et al., 2025; Mahajan et al., 2025; Tian et al., 2025). Moreover, seeds can remain dormant until environmental conditions become favorable, helping plants to survive under stressful conditions (Sajeev et al., 2019). In contrast, seedlings are generally more vulnerable because continued growth during early establishment depends on continuous uptake of water and nutrients, active metabolism, cell expansion, and root and shoot development, which are easily disrupted under stress conditions (Deng et al., 2022; Lewandrowski et al., 2021). Under drought and salinity stress, reduced water availability and osmotic imbalance disrupt cellular metabolism, photosynthesis, and nutrient uptake, leading to reduced root and shoot growth, lower biomass accumulation, and impaired seedling establishment and survival (Begum et al., 2022; Rajabi Dehnavi et al., 2020; Wang et al., 2025). These stresses also induce transcriptional changes associated with photosynthesis, antioxidant and ROS-scavenging systems, hormone signalling, and osmotic regulation, thus influencing seedling adaptation and survival under stress conditions (Ali et al., 2024b; Li et al., 2016). Consequently, abiotic stresses during these early developmental stages strongly influences seedling vigour, establishment success, and subsequent plant survival.

Beyond plant genetics and environmental factors, plant-associated microorganisms can also influence seed germination and seedling establishment (Walsh et al., 2021). These microbes are proposed to be able to affect seed vigour and stress resilience, suggesting that seed traits are shaped not only by plant and environmental factors but also by associated microorganisms (Brunel-Muguet et al., 2025; Matilla, 2025; Otani et al., 2024; Ugarte et al., 2024).

Plant microbiome

Plants host diverse communities of microorganisms across their organs, including roots, leaves, stems, flowers, and seeds. These communities, collectively called the plant microbiome, consist of bacteria, fungi, archaea, and viruses associated with plant tissues (Dastogeer et al., 2020; Liu et al., 2024a). Based on their colonization site, plant-associated microorganisms can be classified as epiphytes, which live on the surface of plant organs, or endophytes, which live inside plant tissues (Bacon & White, 2016). Plants recruit their microbes from many sources, including soil, air, water, and seeds, and the composition of these microbial communities is also influenced by plant genetics, as different genotypes can selectively recruit and shape distinct microbial communities (Compant et al., 2019; Xiong et al., 2021). Among these, the surrounding soil and rhizosphere are major sources of plant-associated microorganisms, where root exudates, including sugars, amino acids, and organic acids, attract microbes that later colonize plant surfaces and tissues, contributing to the establishment of the plant microbiome (Santoyo, 2022).

Plant-associated microbes can also be classified as neutral, harmful, or beneficial based on their effects on plant growth and performance (Trivedi et al., 2020). Neutral microorganisms, known as commensals, benefit from the plant by using it as a habitat or resource without causing obvious positive or negative effects on plant fitness (Sohrabi et al., 2023). Harmful microorganisms, known as plant pathogens, cause diseases and negatively affect plant growth and productivity, including bacteria, fungi, and viruses (Nazarov et al., 2020). In contrast, beneficial microorganisms, most of which are bacteria and fungi, establish mutualistic associations with plants, where both partners benefit and contribute positively to plant growth and performance (Narkhede et al., 2026). Among the diverse members of the plant microbiome, beneficial microorganisms have gained increasing attention due to their important roles in plant health and fitness, as well as their potential applications in sustainable and climate-resilient agriculture (Ali et al., 2024a).

Plant growth-promoting microorganisms (PGPM) are beneficial members of the plant microbiome that enhance plant productivity and stress resilience by improving nutrient and water uptake, increasing antioxidant defences, helping nitrogen fixation and phosphate solubilization, and producing phytohormones, and improving plant resistance to pathogens (Liu et al., 2025b; Nikalje et al., 2026; Tiwari et al., 2025). For example, bacterial genera such as *Bacillus* and *Pseudomonas*, as well as fungal genera such as *Trichoderma* and arbuscular mycorrhizal fungi, have been widely reported to improve plant growth, enhance tolerance to abiotic stresses such as drought and salinity, and suppress plant

pathogens (Li et al., 2025b; Liu et al., 2025b). The beneficial effects of the plant microbiome are largely mediated through microbial metabolites and signalling processes involved in plant-microbe interactions. PGPM produce diverse bioactive compounds, including phytohormones (e.g., auxin/indole-3-acetic acid), volatile organic compounds (VOCs; e.g., acetoin and 2,3-butanediol), antibiotics (e.g., phenazines), siderophores, exopolysaccharides, and enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Compant et al., 2019; Shi et al., 2009; Sowmiya et al., 2025; Yasmin et al., 2021). In addition, beneficial microbes can modulate endogenous plant hormonal pathways, including abscisic acid, auxin, jasmonic acid, salicylic acid, and ethylene signalling (Kour et al., 2024). These microbial metabolites and signalling processes enable communication between plants and associated microorganisms, thereby influencing plant growth, defence responses, and adaptation to environmental stress (Hawkes et al., 2020). Among the diverse mechanisms by which plant-associated microorganisms influence plant physiology, VOCs have emerged as important signalling molecules involved in plant growth and stress responses (Ali et al., 2025).

Microbial volatile organic compounds (VOCs) as signalling molecules in plant-microbe interactions

VOCs have gained increasing attention as long-distance signalling molecules in plant-microbe interactions due to their low molecular weight and high diffusibility (Bitas et al., 2013). These properties allow VOCs to move through air, water, and soil and mediate communication between microorganisms and plants without direct contact (Almeida et al., 2022; Kanchiswamy et al., 2015). Microbial VOCs comprise several major chemical classes, including alcohols, ketones, terpenes, esters, aldehydes, organic acids, and sulfur-containing compounds (Ali et al., 2025). VOCs typically produced by bacteria include acetoin, 2,3-butanediol, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), and ketones such as 2-nonanone, which are frequently reported from *Bacillus* and related bacterial genera. In contrast, VOCs typically produced by fungi include 1-octen-3-ol, 3-octanone, 2-pentylfuran, and diverse terpenes produced by filamentous fungi such as *Trichoderma* and *Aspergillus* (Bennett et al., 2012; Elmassry et al., 2020; Morita et al., 2019).

In natural environments, microorganisms continuously release VOCs during active growth and in response to environmental conditions, nutrient availability, microbial competition, and interactions with plants, insects, and other microorganisms (Misztal et al., 2018; Weisskopf et al., 2021). In addition to mediating plant-microbe interactions, VOCs also play important roles in microbe-microbe communication, particularly in complex soil environments. These volatile

compounds are involved in microbial communication, competition, colonization, and stress adaptation, thereby shaping soil microbial community composition and activity and indirectly influencing plant growth and stress responses (Schulz-Bohm et al., 2017; Schulz-Bohm et al., 2015). Plants also emit VOCs that influence microbial attraction, colonization, and activity in the rhizosphere and phyllosphere, thereby shaping plant-associated microbial communities, while microorganisms can also shape plant VOC profiles (Ali et al., 2025; Sharifi et al., 2022). Together, these VOC-mediated interactions contribute to plant-microbe and microbe-microbe communication in plant-associated environments.

Microbial VOCs influence a wide range of plant physiological and molecular processes, including stress-responsive gene expression, modulation of phytohormone signalling pathways (e.g., auxin, ethylene, jasmonic acid, and salicylic acid), alterations in cellular signalling (e.g., calcium fluxes and ROS production), plant development (e.g., root system architecture and resource acquisition), and plant defence responses (e.g., induction of systemic resistance (ISR) against pathogens) (Fincheira et al., 2021; Tahir et al., 2017a; Tyagi et al., 2018). Through these coordinated effects, VOCs modulate multiple plant regulatory networks, thereby influencing plant growth and stress responses (Narayanasamy & Uthandi, 2024; Türksoy et al., 2025). For example, VOCs produced by *Burkholderia pyrrocinia* CNUC9, including dimethyl disulfide (DMDS) and 2-undecanone, enhanced growth and salinity tolerance in *Arabidopsis thaliana* by improving root architecture, osmotic balance, and antioxidant responses while reducing ROS accumulation and lipid peroxidation under salt stress (Luo et al., 2022). Similarly, VOCs from *Rahnella aquatilis* JZ-GX1, including 2-undecanone and 3-methyl-1-butanol, improved tolerance to iron deficiency by promoting root growth, rhizosphere acidification, iron uptake, and ABA-mediated stress signalling pathways (Kong et al., 2021). Microbial VOCs are increasingly recognized as important mediators of plant growth, defence, and stress responses, with potential applications for improving crop establishment and resilience (Bruisson et al., 2023; Mokh et al., 2025). However, most VOC studies have focused on rhizosphere microbiome, whereas seed-associated microbes and their VOCs remain comparatively understudied despite evidence that they can influence seed germination, seedling establishment, and early stress responses (Marzouk et al., 2021).

Seed microbiome

The seed microbiome is a specialized subset of the plant microbiome that serves as the initial microbial inoculum, forming a close association with seed tissues during early plant development (Chesneau et al., 2022; War et al., 2023). Seed-associated microorganisms occur both on the seed surface and within internal

seed tissues, including the endosperm and embryo, where they can establish stable associations with the host plant (Bacon & White, 2016; Truyens et al., 2015; Xu et al., 2026). Compare to other plant compartments, the seed microbiome is generally less diverse and less abundant due to strong host-mediated selection and the restricted, nutrient-limited environment within seeds (Adeboye et al., 2025; David & Parthasarathi, 2026). Seed microbiomes are typically dominated by a set of common microbial taxa, including bacterial genera such as *Pantoea*, *Enterobacter*, *Bacillus* and *Pseudomonas*, and fungal genera such as *Fusarium* and *Alternaria.* These taxa are commonly shared across plant species and are also capable of colonizing germinated seeds and seedlings, thereby contributing to microbiome establishment during early developmental stages (Johnston-Monje et al., 2021).

A key feature of the seed microbiome is its ability to be transmitted across plant generations, particularly through vertical transmission of endophytes inherited directly from the mother plant via reproductive tissues such as flowers, ovules, and vascular tissues connected to the seeds (Fig. 1) (Berg & Raaijmakers, 2018; Bergna et al., 2018). This transmission enables beneficial microbes to transfer functional traits from parent plants to their offspring, helping to establish early plant-microbe interactions during germination and seedling development (Khan et al., 2025; Shade et al., 2017). In addition to vertical transmission, the seed microbiome is also shaped by horizontal acquisition of microorganisms from the surrounding environment, particularly through flowers, pollinators, and other reproductive tissues during seed development (Fig. 1) (Frank et al., 2017; Shahzad et al., 2018). In addition to floral routes, some seed-associated microorganisms also originate from the rhizosphere and colonize the seeds through systemic plant tissues (Rodríguez et al., 2020; Sessitsch et al., 2023). Consequently, seed microbiome composition is strongly influenced by the environmental conditions experienced by the mother plant, which affect microbial recruitment to seeds (Chen et al., 2026; Fort et al., 2021). For example, Tabassum et al. (2024) showed that soil composition, geo-climatic conditions, and cultivation environments significantly shaped the fonio (*Digitaria exilis*) seed microbiome, while many seed-associated taxa were vertically transmitted across generations. The study further showed that different fonio genotypes shared a heritable core microbiome, suggesting that host genotype also contributes to seed microbiome assembly and inheritance (Tabassum et al., 2024). Similarly, Morales Moreira et al. (2021) further showed that crop type, host genotype, and field environmental conditions significantly influenced bacterial and fungal seed microbiome composition across wheat, canola, and lentil, highlighting the combined roles of host genetics and environmental factors in shaping seed-associated microbial communities. Together, these findings suggest that seed microbiome assembly is shaped by

the combined effects of environmental conditions, microbial transmission routes, and host genetic factors. However, how these factors interact to determine seed microbiome assembly and persistence across generations remains largely unknown.

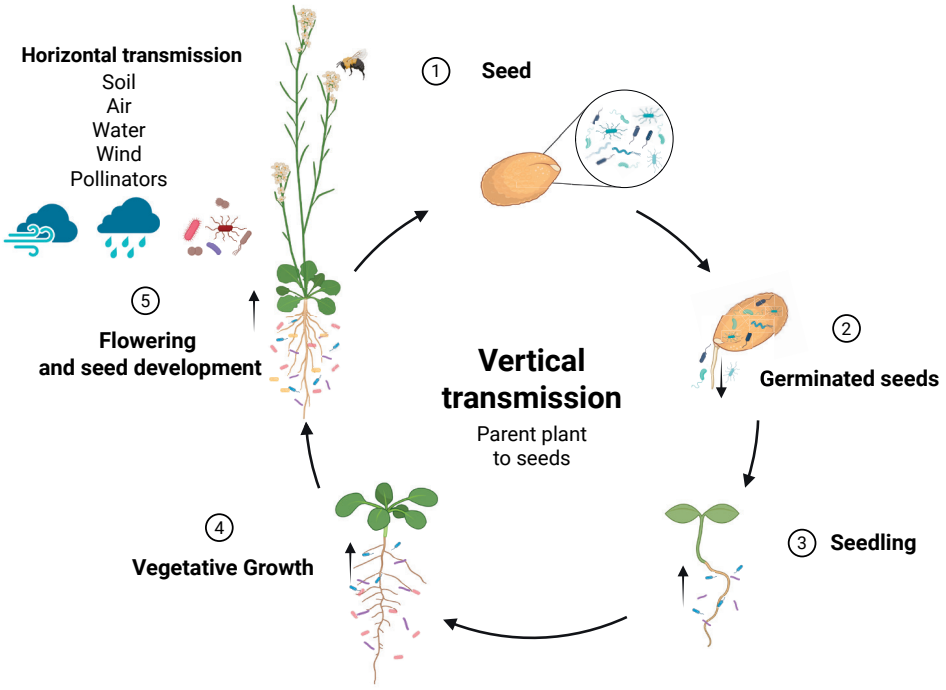


Figure 1: Schematic representation of microbial associations with seeds across plant developmental stages. The figure presents the plant life cycle (Arabidopsis) from the parent plant to seeds with stages of vertical transmission of seed-associated microbiota indicated. This microbiome might persists during germination, seedling establishment, and vegetative growth. In addition to vertical transmission, horizontal transmission from environmental sources such as soil, air, water, and pollinators contributes to the assembly of the seed microbiome. Together, these processes shape the composition and dynamics of seed-associated microbial communities throughout the plant life cycle.

In addition to transmission processes, seed maturation also plays an important role in seed microbiome assembly. During maturation, physiological changes within seeds, including desiccation and nutrient limitation, act as selective filters that shape a relatively limited and specialized microbial community associated with mature seeds (Adeboye et al., 2025; Kim et al., 2022). These conditions determine which microorganisms can survive, persist in dry seeds, and be transmitted to the next plant generation (Gerna et al., 2026). Some members of the seed microbiome are associated with beneficial functions that influence early plant development and adaptation to stress conditions (Hubbard et al., 2012;

Wang & Zhang, 2023). These beneficial roles are supported by various studies showing that seed-associated microbiomes can improve seed germination and seedling growth under both optimal and stress conditions in crops such as maize (Bomfim et al., 2020; Siddique et al., 2022), tomato (Lengrand et al., 2025), and rice (Ahumada et al., 2022; Matsumoto et al., 2021). Moreover, under stressful environmental conditions, this selective process may be enhanced, with mother plants recruiting and transmitting beneficial endophytes to progeny seeds, thereby improving stress adaptation in the next generation (Bergna et al., 2018; Walitang et al., 2018). For example, drought stress in wheat altered progeny seed microbiome composition and enriched Actinobacterial genera such as *Curtobacterium* and *Arthrobacter* in drought-tolerant lines, and these isolates subsequently promoted root growth and drought tolerance in wheat seedlings (Hone et al., 2021).

Seed endophytes are important components of the plant microbiome because they can vertically transmit beneficial functions to the next generation, thereby promoting germination, early seedling growth, and stress tolerance, partly through microbial VOC production (Figueiredo dos Santos et al., 2021; Li et al., 2025a; Marzouk et al., 2021). Existing studies suggest that seed endophyte-derived VOCs can influence germination and seedling growth, as well as suppress plant pathogens through diverse bioactive compounds with antimicrobial and plant growth-promoting activities. For example, seed-associated *Bacillus* spp. produce VOCs such as 2-heptanone, pyrazine, naphthalene, benzenamine, and benzothiazole that promote seedling growth and suppress plant pathogens through antifungal activity by suppressing mycelial growth and sclerotia formation, inducing ROS accumulation, and reducing pathogen virulence (Marzouk et al., 2021; Massawe et al., 2018). Similarly, VOCs produced by seed endophytic *Pantoea* and *Pseudomonas* spp., including DMDS and DMTS, promoted seed germination and seedling growth while suppressing fungal pathogens (Tyc et al., 2020). Despite these findings, studies specifically investigating VOCs released by seed endophytes remain limited, particularly during early plant developmental stages and under abiotic stress. Furthermore, the molecular mechanisms underlying plant responses to seed-derived VOCs are still poorly understood.

Arabidopsis thaliana as a model plant for plant-microbe interaction studies

Arabidopsis thaliana is a powerful and widely used model for plant-microbe interaction studies due to its well-characterized microbiome, extensive genetic resources, and the availability of numerous accessions, including both mutant and wild-type lines, which make genetic manipulation straightforward and allow

in-depth study of plant responses to microbial signals (Koornneef & Meinke, 2010; Poupin et al., 2023). Its well-documented responses, including hormone signalling pathways and stress-related gene expression, together with abundant genomic and biological information, support mechanistic studies of the molecular processes underlying plant-microbe interactions (Mauch-Mani & Slusarenko, 1993; Ortíz-Castro et al., 2009). In addition, its small size and simple growth requirements make it suitable for controlled experiments, while its rapid life cycle enables studies of early development and transgenerational processes, such as the vertical transmission of microbiomes across generations (Woodward & Bartel, 2018).

In addition to laboratory-based studies, the availability of diverse natural populations of *Arabidopsis thaliana* supports research under ecologically relevant conditions (Mitchell-Olds & Schmitt, 2006). Among these populations is the from *Arabidopsis thaliana* populations from the Iberian Peninsula. This population exhibits high genetic diversity and strong geographical structuring, with significant population differentiation and isolation by distance Picó et al. (2008). Their distinct genetic clusters are distributed across regions, reflecting local adaptation and complex evolutionary history. Such characteristics make natural populations a powerful resource for investigating how plant-microbiome interactions vary across environments and generations (Barnes et al., 2025; Mertin et al., 2023). In addition, seeds collected from nature provide a valuable source for the isolation of endophytic microorganisms, which may serve as beneficial and culturable candidates for improving plant growth and stress tolerance, as these microbes are likely adapted to diverse and often harsh environmental conditions in natural habitats (Waqas et al., 2025).

The outline of thesis

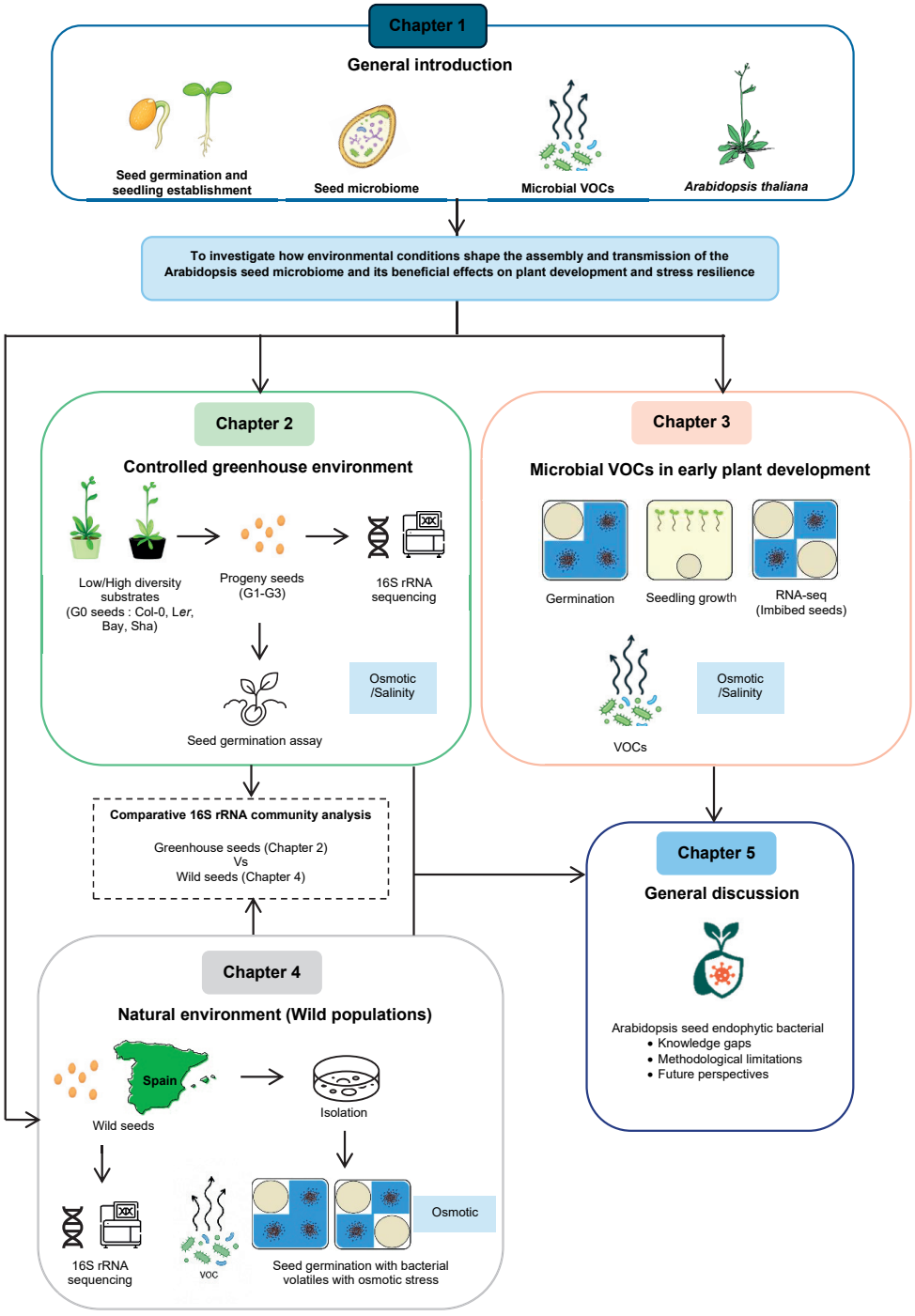
This thesis focuses on seed microbiome recruitment under different growth substrates in controlled greenhouse conditions, its vertical transmission across generations, and the role of microbial VOCs in regulating seed germination and seedling growth under abiotic stress It also examines bacterial endophyte communities associated with wild plant populations and evaluates the effects of different maternal environments, particularly comparing plants grown in greenhouse and natural (wild) conditions. By integrating ecological, physiological, and molecular approaches, this work provides insight into the structure, transmission, and potential roles of seed-associated microbial communities on seed germination and seedling establishment. The outline of the thesis is also summarized in Fig. 2.

Chapter 2 of this thesis examines how growth substrate influences seed microbiome composition and seed germination across three successive generations of *Arabidopsis thaliana*. Plants from different accessions were grown on four types of substrates with varying levels of microbial diversity over three generations under controlled greenhouse conditions. Progeny seeds were tested for germination under salinity and osmotic stress to assess the effects of growth substrate on germination performance and seed endophytic bacterial communities. This chapter focuses on the dynamics of microbial transmission and recruitment, highlighting how variation in substrate microbial diversity and plant genotype shapes seed-associated microbial communities and influences plant performance.

Chapter 3 of this thesis investigates the effects of bacterial volatile organic compounds (VOCs) produced by seed endophytic bacteria isolated from wild cabbage, *Pantoea agglomerans* E44, on the germination and seedling growth of *Arabidopsis thaliana* under abiotic stress, including salinity and osmotic stress. In addition to phenotypic analyses, this chapter incorporates transcriptome analyses to examine the molecular responses of imbibed seeds prior to germination upon exposure to bacterial VOCs under control and salinity stress conditions, providing insight into the underlying signalling and regulatory mechanisms.

Chapter 4 aims at the identification of conserved microbial taxa in *Arabidopsis thaliana* wild populations, collected from different locations in Spain, and greenhouse-grown plants (data from Chapter 2). It assesses how maternal environments influence the seed-associated microbiome composition and evaluates the potential of seeds collected from wild plants as reservoirs of beneficial microbes that contribute to seed germination. In addition, culturable seed endophytes are isolated and identified, and their ability to promote seed germination under drought stress is evaluated, with a particular focus on the role of VOCs.

Finally, **Chapter 5** presents a general discussion from all chapters and addresses remaining knowledge gaps and methodological limitations, while outlining directions for future research. In addition, this chapter discusses the potential application of seed endophyte-based strategies in sustainable agriculture, particularly for improving plant establishment and resilience under environmental stress.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General introduction
Seed germination and seedling establishment
Seed microbiome
Microbial VOCs
Arabidopsis thaliana
To investigate how environmental conditions shape the assembly and transmission of the Arabidopsis seed microbiome and its beneficial effects on plant development and stress resilience
Chapter 2
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Chapter 4
Natural environment (Wild populations)
Spain
Wild seeds
Isolation
16S rRNA sequencing
VOC
Seed germination with bacterial volatiles with osmotic stress
Osmotic

Figure 2: Schematic overview of the thesis structure and experimental framework. Chapter 1 provides a general introduction to seed germination and seedling establishment, the plant and seed microbiome, microbial volatile organic compounds (VOCs), and plant-microbe interactions. Chapter 2 investigates seed microbiome recruitment under greenhouse conditions using different growth substrates, vertical transmission across three generations (G1-G3), and the influence of growth substrates on seed germination under abiotic stress conditions. Chapter 3 examines the role of microbial VOCs in seed germination under abiotic stress and investigates transcriptional regulation in imbibed seeds using RNA-seq analysis. Chapter 4 explores seed microbiome composition in natural Arabidopsis populations and includes a comparative analysis of 16S rRNA amplicon sequencing data between greenhouse-derived seeds (Chapter 2) and wild seeds (Chapter 4). Chapter 5 summarizes the main findings, discusses ecological implications, and highlights future research perspectives.

Chapter 2

Growth substrate and host genotype influence seed endophytic bacterial communities and seed germination performance in *Arabidopsis thaliana*

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ABSTRACT

Seed-associated microbiomes play a key role in the early stages of plant development and stress resilience, but how host growth substrate and host genotype shape seed microbial communities and germination remains poorly understood. We investigated four *Arabidopsis thaliana* accessions grown on substrates varying in microbial diversity over multiple generations. Seed germination assays under abiotic stresses were performed to assess stress resilience, while 16S rRNA sequencing was used to profile bacterial seed endophytes. Our results showed that seed microbiomes were primarily shaped by a vertically inherited core community dominated by Firmicutes and Bacteroidota, with a smaller fraction influenced by horizontal transmission. Beneficial lactic acid bacteria, including *Carnobacterium* and members of the order *Lactobacillales*, were enriched in seeds from non-sterile field soils, potentially promoting germination and stress tolerance. Substrate-derived microbial transmission was only evident in seeds continuously grown on rockwool over numerous cycles, suggesting that prolonged cultivation may be required for microbes from rockwool to integrate into seeds. Overall, our data showed that the seed microbiomes are shaped by the interplay of vertical and horizontal microbial transmission, host genotype, and environmental conditions, which together influence germination performance and stress resilience. These findings help to understand the role of seed microbiota for seed and plant performance and survival, and provide a basis for new microbial-assisted plant breeding strategies aimed at selecting plants capable of recruiting beneficial microbes, offering a sustainable approach to enhance seed vigour, stress tolerance, and crop resilience.

Keywords: Seed germination, Abiotic stress, Seed microbiome, *Arabidopsis thaliana*, Vertical transmission, Horizontal transmission

INTRODUCTION

Seed germination is a critical stage in the plant life cycle, establishing the basis for seedling establishment, crop productivity, and, eventually, global food security (Finch-Savage & Bassel, 2016; Nabuuma et al., 2022). With the increasing severity of climate change, seeds are more frequently exposed to abiotic stresses such as drought, heat, and salinity, which can impair germination, seedling vigor, and lead to reduced crop yields (Maity & Pramanik, 2013). These challenges highlight the urgent need to identify factors that can sustain seed performance under adverse environmental conditions. Recently, the microbial communities associated with seeds, known as the seed microbiome, have gained more attention for their ability to support germination, seedling establishment, and overall plant performance, particularly under stress conditions. These communities consist of host-adapted microorganisms capable of functioning under challenging conditions, acting as the primary inoculum for the plant's microbiome (Hone et al., 2021; Sun et al., 2023; War et al., 2023). Numerous bacterial taxa have been identified within seed tissues of different plant species, including Arabidopsis, such as *Pseudomonas*, *Pantoea*, *Xanthomonas*, and *Sphingomonas* (Poupin et al., 2023). These seed microbes can promote plant growth through phytohormone production, enhance nutrient uptake via nitrogen fixation and phosphate solubilization, improve stress resilience, and suppress pathogens (Sharma et al., 2025). As climate change worsens and the need for sustainable crop production grows, the seed microbiome is therefore seen as a promising, low-input strategy to enhance plant resilience and reduce the use of chemical fertilizers and pesticides (Romão et al., 2025). Understanding how seed-associated microbial communities are assembled, maintained, and transmitted across generations is therefore essential for employing their potential to improve crop performance under diverse and changing environmental conditions.

The host environment plays a crucial role in shaping both seed germination, performance and the composition of the seed-associated microbiome (Abdelfattah et al., 2023; Rodríguez et al., 2020). Host environmental factors, including soil nutrient availability, temperature, moisture, and stress conditions, were shown to influence seed nutrient content, hormone balance, and physical traits, eventually determining germination success (Humphries et al., 2018; Javaid et al., 2022). Beyond their effects on seed physiology, host growing conditions also shape microbial communities through vertical and horizontal transmission routes (Shade et al., 2017). Vertical transmission is the direct inheritance of microbes, mainly endophytic, from the parent plant's internal tissues to its seeds via the vascular system or floral meristems. In contrast, horizontal transmission occurs when seeds acquire microbes from external sources such as soil, air, irrigation

water, plant surfaces, pollinators and other insects. The interaction between vertical and horizontal transmission routes ultimately shapes the diversity, composition, and stability of the seed microbiome (Frank et al., 2017; Shade et al., 2017; Shahzad et al., 2018). Through both transmission routes, certain microbial taxa, particularly bacterial endophytes, are selectively recruited and inherited across generations, resulting in seed-associated microbial communities that are generally less diverse than those found in other plant compartments such as the rhizosphere or phyllosphere (Nelson, 2018). Despite this lower diversity, these communities often harbour a specific and stable subset of microbes, known as the core seed microbiome, that persist across generations and can confer beneficial traits enhancing seed and seedling resilience (Johnston-Monje et al., 2021; Simonin et al., 2022).

Among host environmental factors, soil plays a crucial role in shaping the seed microbiome, as it contains diverse and abundant microbial communities that serve as the source of seed-associated microbes (Walsh et al., 2021). Variations in soil properties and microbial communities can shape the composition and diversity of seed microbiomes, leading to differences in germination success across environments (Dumigan & Deyholos, 2024; Rodríguez et al., 2020). For example, Rodríguez et al. (2020) demonstrated that growing *Setaria viridis*, a green foxtail, for multiple generations in sterile soil led to a reduction in seed bacterial microbiome diversity and shifts in community composition, which corresponded with declines in seed vigour and germination performance in later generations. Similarly, Eldridge et al. (2021) showed that variations in soil microbiome composition and diversity significantly affected seed germination rates and seedling vigour, with effects varying according to both soil type and plant species. Furthermore, recent work on *Glycine clandestina*, a wild relative of soybean, revealed that seeds grown from native soil retained a higher diversity and more consistent bacterial communities across generations compared to those grown in less diverse media (Chandel et al., 2022a). These studies highlight soil as a crucial reservoir for seed microbial communities and a major contributor to early plant development. In addition to environmental factors, plant genotype also plays a vital role in shaping seed microbiome recruitment and germination performance. Studies in cereals (Malacrino et al., 2022) and pumpkin (Adam et al., 2018) show that host genetic variation influences seed microbial communities. Research on *Brassica napus* (Rochefort et al., 2019a) and *Digitaria exilis* (Tabassum et al., 2024) further highlights that genotype and environment jointly affect seed microbiota diversity and structure. Liu et al. (2024) demonstrated that hybrid rice varieties exhibit improved seed germination linked to a more diverse seed endophytic microbiome than their parental lines. These studies showed the

important role of host genotype and environmental factors in shaping seed microbiome composition and germination performance.

However, the mechanisms underlying how soil environment and host genotype interact to shape seed microbial communities, their transmission, and seed germination performance remain poorly understood. Addressing this knowledge gap is crucial for harnessing the seed microbiome in order to improve crop productivity and stress resilience. To address these questions, we used *Arabidopsis thaliana*, a model species with a small genome and short life cycle suitable for multi-generational experiments under controlled conditions. Seeds from four accessions were grown on four substrates varying in microbial diversity, allowing us to assess the effects of host growth substrate and plant genotype on seed microbiome assembly, vertical and horizontal transmission, and seed germination under abiotic stresses. These insights provide a basis for designing seed microbiome strategies that improve crop resilience and yield for sustainable agriculture.

MATERIALS AND METHODS

Generation zero (G0) seeds

Seeds from four different *Arabidopsis thaliana* accessions were used in this experiment: Columbia-0 (Col-0), Shahdara (Sha), Landsberg *erecta* (L*er*), and Bayreuth (Bay). All plants were cultivated on rockwool in a climate chamber maintained at 20°C during the day and 18°C at night, with a 16-hour light/8-hour dark photoperiod. Seeds from Col-0 and L*er* were harvested in December 2021, while Sha and Bay were harvested in March 2022. Prior to use, the seeds were stored in a controlled cabinet at 20°C and 30% relative humidity.

Growth substrates

Four different substrates were used in this experiment, namely sterile field soil, non-sterile field soil, potting substrate and rockwool. Field soil, referred to as "MiCRop" soil, was collected in 2014 from an organic field in the Nergena area, Bennekom, the Netherlands (coordinates: 51.996250, 5.659375) described in Berdaguer et al. (2024). The soil was then packed and sealed in plastic bags. Gamma-sterilized soil was derived from the same "MiCRop" field soil and sterilized using gamma radiation. The irradiation performed with a dose of 25 kGy by STERIS located in Ede, the Netherlands, effectively eliminated microbial life while preserving the soil's physical structure. The sterilized soil was sealed and stored in two-layer plastic bags to prevent re-contamination. Both the packed field soil and gamma-sterilized soil were stored at 4°C before being used in the

experiment. Commercial potting substrate (Lensli BV, NL), primarily composed of peat and perlite. Rockwool (Grodan ROCKWOOL B.V., NL), made from basalt rock. This substrate, irrigated with Hyponex nutrient solution, is commonly used in Wageningen University & Research (WUR) greenhouse facilities for Arabidopsis propagation and was also the substrate on which generation zero seeds were produced.

Production of successive generation seeds (G1, G2, and G3)

G0 Arabidopsis seeds from all accessions were cultivated on the four distinct substrates, as described above, to produce first-generation seeds (G1). Each treatment was arranged in a randomized block design with four biological replicates, and each replicate consisting of four plants. Plants were grown under controlled conditions in a WUR greenhouse with a 16-hour light / 8-hour dark photoperiod, at a temperature of 20°C (day) and 18°C (night), with 60% relative humidity. Additionally, plants were watered with Hyponex fertilizer (Hyponex Japan, Japan). Once the plants reached maturity and the siliques had dried and turned brown, G1 seeds were harvested separately from each biological replicate in November 2022. The seeds were then stored in a controlled cabinet at 20°C and 30% relative humidity until the next generation was sown.

To study the inheritance of the seed endophytic microbiome across generations, G1 seeds were sown in the same four substrates under identical growth conditions, except that plants grown in field soil, sterilized soil, and potting substrate were watered with tap water, while those grown in rockwool continued to receive Hyponex fertilizer. All plants were grown to maturity, and second-generation seeds (G2) were harvested in April 2023, following the same storage procedure as described above. To produce the third-generation seeds (G3), G2 seeds were sown, and the propagation process was repeated using the same methodology as for G2 seed production. G3 seeds were then harvested in September 2023, and the same storage conditions were applied.

Dry seed surface sterilization for germination assay

Before performing the seed germination assay, seeds were surface sterilized with chlorine gas. In the fume hood, opened Eppendorf tubes containing the seeds were placed inside a closed desiccator together with a glass beaker containing 100 ml of bleach (sodium hypochlorite) and 3 ml of 37% hydrochloric acid to generate chlorine gas. Seeds were incubated with chlorine gas for 5 hours. After sterilization, seeds were kept in Eppendorf tubes and stored in a controlled cabinet at 20°C and 30% relative humidity prior to use.

Seed germination assay

Preliminary germination assays were performed with G1 seeds under varying NaCl and D-mannitol concentrations to determine suitable stress levels (Supplementary Figs. 1-4). Concentrations of 100 mM NaCl and 400 mM D-mannitol were selected as optimal salinity and osmotic stress treatments, respectively, as they induced sufficient stress to differentiate responses among substrates and accessions. Seeds from three generations (G1-G3) germinated under these stress and control conditions. Seeds used in this experiment were two months post-harvest and had been stored under controlled conditions at 20°C and 30% relative humidity prior to use. Surface-sterilized seeds were sown on two layers of blue germination paper placed in a plastic tray containing 40 mL of either NaCl solution (100 mM), D-mannitol solution (400 mM), or demineralized water (control). Each tray contained six germination samples with approximately 50 seeds per sample, and four biological replicates were used. Trays were then incubated at 22°C for seven days under continuous light conditions. Germination progress was monitored by capturing images twice daily using a Nikon D80 camera (Nikon, Japan) equipped with a 60 mm macro lens, and the resulting images were used for quantitative germination analysis.

Germination analysis

Germination images, as described before, were processed using ImageJ, which automatically detects germinated seeds based on radicle emergence. Germination parameters, including the maximum germination percentage (Gmax) and the time to reach 50% of maximum germination (T50), were then calculated using the GERMINATOR software package (Joosen et al., 2010). Statistical analysis of germination parameters was performed in RStudio. Differences among datasets were assessed using one-way ANOVA, followed by Fisher's least significant difference (LSD) test for multiple comparisons at $p < 0.05$.

Wet seed surface sterilization for seed endophytic microbial DNA extraction

Prior to DNA extraction, Arabidopsis seeds were wet surface-sterilized to remove epiphytic microorganisms from the seed surface. All procedures were performed under a laminar flow cabinet to maintain sterility. A total of 50 mg of Arabidopsis seeds were placed in a 2 mL Eppendorf tube, and 1 mL of 0.1% sodium hypochlorite mixed with 0.1% Tween 80 was added. The tube was gently tapped to mix, ensuring minimal foaming. The seeds were incubated in a sterilizing solution for 1 minute. Centrifuged briefly to precipitate, then carefully removed the sterile solution using sterile filter-tips. The seeds were then washed five times with 1 mL of sterile demineralized water per wash. A new sterile pipette tip was used for each wash to prevent contamination. To confirm effective sterilization, the final

wash water was incubated in 1/10th tryptic soy broth agar (TSBA) (5.0 g/L NaCl; 1.0 g/L KH_2PO_4; 3 g/L Oxoid Tryptic Soy Broth; and 20 g/L BACTO agar, pH 6.5) for seven days, ensuring that no bacterial or fungal growth appeared.

Microbial DNA extraction from seeds

Fifty milligrams of sterilized seeds were transferred to a sterile mortar, and 1 mL of 10 mM phosphate buffer (P-buffer) (1.361 g/L KH_2PO_4 in 1 L of demineralized water, pH 6.5) was added. The seeds were manually grounded using a sterile pestle until fine. An additional 4 mL of P-buffer was then added to the mortar and briefly mixed. The mixture of ground seeds and P-buffer was transferred to a 50 mL sterile Greiner tube and incubated at 20°C for 45 minutes with shaking at 180 rpm to obtain the seed suspension. Half of this mixture was centrifuged at 15000xg, the pellet resuspended to a volume of 250µl and stored at -70°C for later DNA extraction. The 250 µL of this suspension per replicate was used for microbial DNA extraction, with each treatment performed in four biological replicates. Microbial DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, NL) following the manufacturer's instructions. DNA was quantified using a Nanodrop (Thermo Fisher Scientific Inc., USA).

Microbial DNA extraction from growth substrates

Microbial DNA was extracted from substrates collected prior to planting for each generation, representing the initial pre-planting microbial communities. For rockwool, 250 mg was torn into small fragments and ground in a sterile mortar with 5 mL of sterilized 10 mM P-buffer (pH 6.5). The mixture was then transferred to a 50 mL sterile Greiner tube and incubated at 20°C for 45 minutes while shaking at 180 rpm. Following incubation, the mixture was filtered using a sterile 40 µm pore-size cell strainer (Corning, USA) to obtain the rockwool suspension for DNA extraction. A volume of 250 µL of rockwool suspension or 250 mg of field soil, gamma-sterilized field soil, or potting substrate per replicate was used for microbial DNA extraction, with each treatment performed in four technical replicates. DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, NL) following the manufacturer's instructions. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

16S rRNA library preparation and sequencing for microbial communities

16S rRNA gene amplicon libraries were prepared and sequenced by Novogene (UK) to analyze the microbial communities of seeds and substrates. For seed microbial communities, the V5–V7 region of the 16S rRNA gene was amplified using the primers 799F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC).

For substrate microbial communities, the V3–V4 region was amplified using the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). To minimize non-target amplification of mitochondrial and plastid DNA, peptide nucleic acid (PNA) clamps were used, including mPNA (GGCAAGTGTTCTTCGGA) and pPNA (GGCTCAACCCTGGACAG). Samples were sequenced on a NovaSeq 6000 (Illumina) platform, generating approximately 100,000 raw sequence reads per sample for seed samples and approximately 30,000 raw sequence reads per sample for substrate samples, ensuring sufficient coverage for downstream microbial community analysis.

Amplicon sequence processing and analysis

Raw 16S rRNA gene sequencing data were processed using NG-Tax 2.0 (v2.2.10) (Poncheewin et al., 2019), with read lengths for both forward and reverse reads set to 140 bp (-for_read_len and -rev_read_len) and a minimum detection threshold (-minPerT) of 0.01. Amplicon sequence variants (ASVs) were identified on a per-sample basis, and taxonomic classification was performed using the SILVA 138.1 reference database for the 16S rRNA gene (Quast et al., 2013). The resulting NG-Tax output was imported into RStudio (v4.4.1), where a phyloseq object was constructed by integrating the ASV table, phylogenetic tree, and sample metadata using the phyloseq package (v1.48.0) (McMurdie & Holmes, 2013). ASVs assigned to taxonomic classes Cyanobacteria and Rickettsiales were excluded from all samples due to their known associations with plant-derived sequences. Additionally, five samples were removed due to insufficient read counts, specifically: one L*er* accession sample grown in rockwool, two Bay accession samples from rockwool and gamma-sterilized field soil conditions, and two Sha accession samples from gamma-sterilized field soil and non-sterile field soil conditions, respectively. Taxa present in only a single sample across the dataset was also excluded to ensure consistency in downstream analyses.

Alpha diversity was calculated using the observed richness index via the estimate_richness function, while beta diversity was assessed through non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity using the metaMDS function. Unique taxa were visualized with Venn diagrams generated using the venn (v1.12) and VennDetail (v1.20.0) packages. Differential abundance analysis was conducted with the DESeq2 package (v1.44.0) (Love et al., 2014). General data handling was performed using the tidyverse suite (v2.0.0) (Wickham et al., 2019), and visualizations including bar plots, box plots, and scatter plots were generated using ggplot2 (Villanueva & Chen, 2019).

RESULTS

Germination performance under osmotic and salinity stress is modulated by accession, growth substrate, and generation

To assess the influence of growth substrate on seed germination performance under abiotic stress conditions, seeds of four *Arabidopsis thaliana* accessions (Col-0, L*er*, Bay, and Sha) were evaluated across three successive generations (G1, G2, and G3). Seeds were produced on four growth substrates, including rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Under control conditions the Gmax exceeded 95% across all substrates, generations, and accessions, with no significant differences observed (Supplementary. Fig.5 and 6). T50 was consistently the lowest in rockwool-derived seeds, indicating faster germination. Under osmotic stress, germination responses vary by genotype, generation, and parental growth substrate. G1 seeds, from Col-0 and L*er* on non-sterile field soil showed significantly reduced Gmax compared to other substrates, while rockwool-derived seeds exhibited the highest germination. This trend continued in G2, with rockwool-grown seeds outperforming others across all accessions. In G3, substrate effects became more variable. Col-0 seeds from non-sterile field soil displayed significantly higher germination percentages compared to those derived from gamma-sterilized field soil (Fig. 1). A similar pattern but not significant was observed in L*er* (Supplementary. Fig.5). In contrast, G3 seeds of Bay and Sha accessions exhibited consistently low germination under osmotic stress across all generations and substrates, indicating higher sensitivity to this stress condition (Supplementary Fig. 5). Corresponding T50 values reflected these trends, with rockwool-derived Bay and Sha seeds in G1, and Col-0 seeds in G2, showing higher T50, likely due to higher germination percentages requiring more time to reach 50%. No significant T50 differences were observed in G3 seeds (Supplementary Fig. 6).

Under salinity stress conditions (100 mM NaCl), germination was reduced in all accessions compared to controls (Supplementary Fig. 5). In G1, rockwool-derived Col-0, L*er*, and Bay seeds exhibited the highest germination percentages, while those from non-sterile field soil showed the lowest. In contrast, Sha seeds from the potting substrate had the lowest Gmax among the treatments. In G2, overall germination declined, particularly in Col-0 and Bay, potentially reflecting the absence of nutrient supplementation during plant growth. Despite this, rockwool-derived seeds maintained the highest germination across accessions, with non-sterile field soil seeds being consistently the lowest. This trend persisted in G3, with rockwool-derived seeds showing significantly higher germination rate, while Sha seeds from non-sterile field soil outperformed those from potting substrate and gamma-sterilized soil (Supplementary Fig. 5).

Corresponding T50 values reflected the observed germination trends. In G1, seeds from non-sterile field soil exhibited higher T50, consistent with slower germination with reduced Gmax. No significant differences in T50 were found in G2 for Col-0, L*er*, or Bay; however, Sha seeds from rockwool exhibited the lowest T50 (Supplementary Fig. 6). Particularly, G1 plants were irrigated with a Hyponex nutrient solution, while G2 and G3 plants received tap water in the greenhouse, which may partly explain the observed generational differences in seed performance. These findings indicate that germination performance is influenced by accession, growth substrate, and nutrient availability.

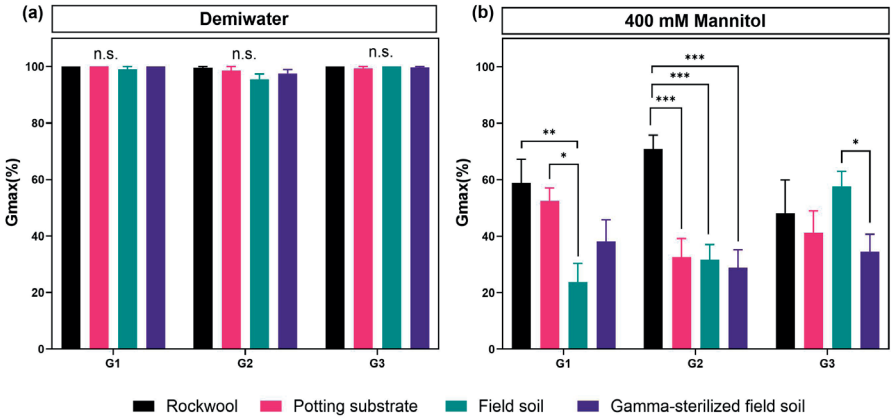


Figure 1: Germination percentage of G1-G3 *Arabidopsis thaliana* Col-0 seeds across different growth substrates under control and osmotic stress conditions. Seeds were cultivated on four substrates, including rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Germination was assessed under (a) control conditions using demineralized water and (b) osmotic stress induced by 400 mM D-mannitol. Values represent means ± standard error (s.e.) from four biological replicates. Statistical significance was assessed using one-way ANOVA followed by the LSD post hoc test. Significance is indicated as follows: ns, not significant ($P > 0.05$); * significant (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$).

Seed microbiome composition is stable across accessions, generations and substrates

To investigate the structure and composition of seed endophytic bacterial communities in *Arabidopsis thaliana*, we performed 16S rRNA gene amplicon sequencing on both G0 and G3 seeds collected from multiple accessions cultivated under different substrate conditions. Taxonomic profiles were generated at the genus level. The top 10 most abundant genera across all samples were visualized in a stacked bar plot (Fig. 2a). The results revealed that the endophytic communities were largely conserved across generations (G0 vs. G3) and accessions, suggesting the presence of a core microbiota that is maintained through seed transmission. Across all samples, the dominant bacterial phyla were *Bacteroidota* and *Firmicutes*.

Among the top 10 genera, *Muribaculum* was the most prevalent across all seed samples, regardless of accession or generation. Other highly abundant genera included *Faecalibaculum*, *Carnobacterium*, *Lactobacillus*, and *Limosilactobacillus*, along with the uncultured genera within the families *Lachnospiraceae* and *Oscillospiraceae* that are classified only at the family level because their genera have not been cultured. These taxa were consistently detected across accessions and generations.

Alpha diversity of seed endophytic bacterial communities, which quantifies the number of unique taxa (richness) within individual samples and provides insight into the complexity of microbial communities, was evaluated using the Observed Diversity Index (Fig. 2b). Significant differences in observed richness were detected among substrate types within specific accessions. In the Col-0 accession, G3 seeds from gamma-sterilized field soil exhibited significantly higher richness of taxa compared to those from non-sterile field soil. On the other hand, in the L*er* accession, seeds from rockwool showed significantly greater richness than those from gamma-sterilized field soil. However, no significant differences were observed in the Bay and Sha accessions across substrate types, suggesting that the influence of growth substrate on seed microbial richness is mainly dependent on plant accession.

To evaluate differences in microbial community composition across treatments and accessions, beta diversity was analyzed using non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity (Fig. 2c). The NMDS revealed a high degree of overlap among samples, with no distinct clustering patterns by accession or substrate. This observation was supported by permutational multivariate analysis of variance (PERMANOVA), which explained 29.97% of the variation in community composition ($R^2 = 0.2997$). However, the model did not reveal any statistically significant differences in community structure ($F = 1.23$, $p = 0.192$; permutations = 999). These findings suggest that while alpha diversity (richness) can vary depending on accession and substrate, the overall beta diversity of seed endophytic bacterial communities remains relatively stable across different treatments and conditions.

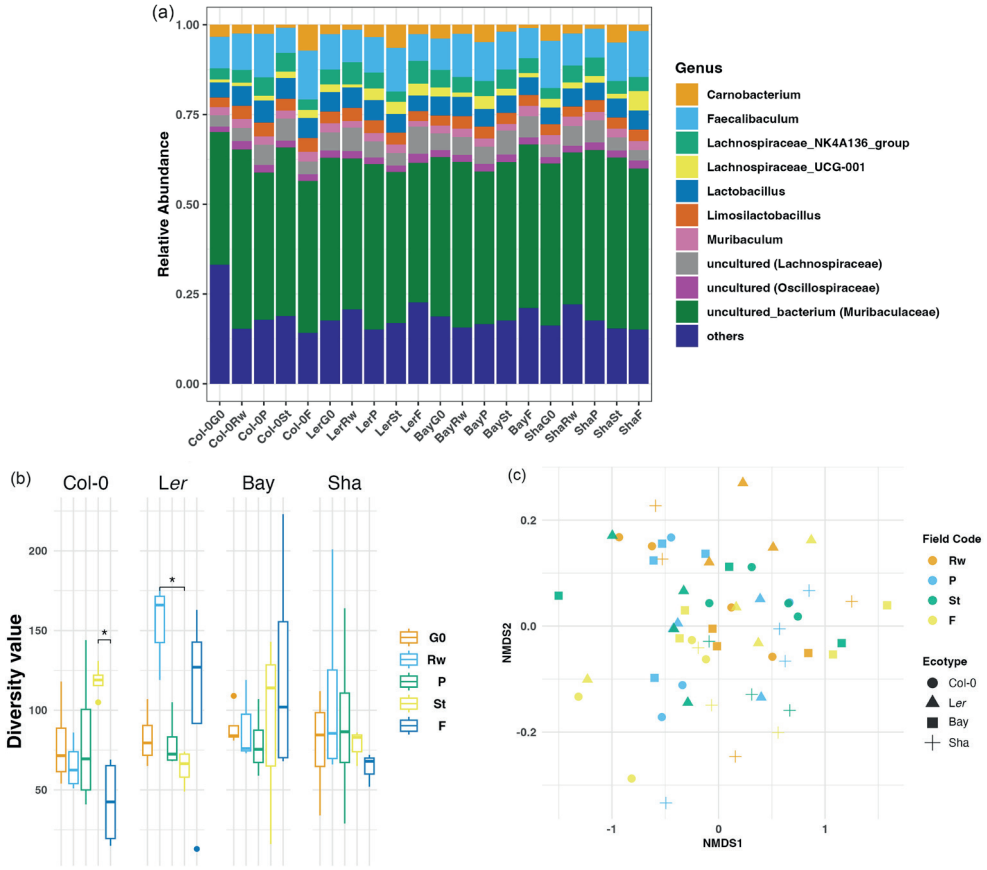


Figure 2: Structure and composition of seed endophytic bacterial communities in *Arabidopsis thaliana* across generations, accessions, and growth substrates. (a) Relative abundance of the ten most dominant bacterial genera (family level shown in parentheses) in G0 and G3 seeds from four accessions (Col-0, L*er*, Bay, and Sha) cultivated on rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Genera outside the top ten in overall abundance were grouped under "Other" for visualization clarity. Taxa labelled as "uncultured" were identified only by sequencing and cannot be assigned to formal genus, but are reported at higher taxonomic ranks (e.g., family). (b) Alpha diversity is measured using the Observed Diversity Index. Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$. (c) Beta diversity of seed bacterial communities visualized using non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity. Differences in community composition were assessed by PERMANOVA (R^2 = 0.2997; F = 1.23; P = 0.192; permutations = 999).

Conserved core bacterial endophytes across generations, substrates and genotypes

To investigate the conservation of seed endophytic bacterial communities, we analysed overlaps between G0 and G3 seeds grown on different substrates across four *Arabidopsis thaliana* accessions using Venn diagrams (Fig. 3). When examined

individually within each accession, the majority of bacterial genera were consistently shared between G0 and G3 seeds, suggesting a strong conservation of the seed microbiome despite environmental differences. Specifically, 24 out of 49, 27 out of 51, 28 out of 48, and 23 out of 41 genera were shared across all seed samples of Col-0, L*er*, Bay, and Sha, respectively. These core genera represented approximately 48.98%, 52.94%, 58.33%, and 56.10% of the total genera identified within each respective accession, representing the dominant portion of the seed endophytic microbiome. In contrast, a relatively small number of bacterial genera were uniquely associated with specific substrates within each accession, ranging from 1 to 7 unique genera per treatment. In Col-0, substrate-specific taxa included *Azospirillum* in G0 seeds, members of *Firmicutes* and *Cyanobacteria* in gamma-sterilized soil, and *Desulfovibrionaceae* in potting substrate (Supplementary Tables 1-4). L*er* seeds from non-sterile field soil and rockwool harbored distinct taxa such as *Marvinbryantia*, *Oscillospiraceae*, and *Clostridia*. For Bay, the majority of unique genera were found in field soil, including *Parabacteroides*, *Lachnospiraceae FCS020 group*, and *Rikenellaceae RC9 gut group*. Sha-specific taxa were mostly detected in potting substrate and rockwool, including *ASF356*, *Muribaculaceae*, and *Desulfovibrionales*. Despite this variation, these substrate-specific taxa represented only a minor fraction of the overall bacterial community (Fig. 3a-d; Supplementary Tables 1-4).

To investigate the existence of a conserved core microbiome independent of growth conditions, we selected the core genera from each accession and analysed their overlap across all accessions and substrate treatments. A total of 20 out of 30 genera (66.7%) were consistently detected across all accessions and substrate treatments, defining a conserved "core" seed endophytic microbiome (Fig. 3e). These conserved genera were mainly associated with the phyla Firmicutes and Bacteroidota. They included members of the families *Muribaculaceae*, *Prevotellaceae*, *Bacteroidaceae*, *Rikenellaceae*, *Marinifilaceae*, *Helicobacteraceae*, *Deferribacteraceae*, *Erysipelotrichaceae*, *Lactobacillaceae*, *Carnobacteriaceae*, *Lachnospiraceae*, and *Oscillospiraceae*. In contrast, several bacterial genera exhibited accession-specific patterns, appearing in only one or a subset of accessions. For example, the genus *Roseburia* and a representative from the phylum Desulfobacterota were specific to the Bay accession. Other genera, such as *Blautia*, *Parasutterella*, and *Desulfovibrio*, were found exclusively in L*er*, Bay, and Sha, but were absent from Col-0. Additional genera were uniquely detected in combinations of two or three accessions (Supplementary Table 5). These findings highlight a highly conserved core seed microbiome, along with a limited set of accession-specific taxa, that persists across seed generations and growth substrates.

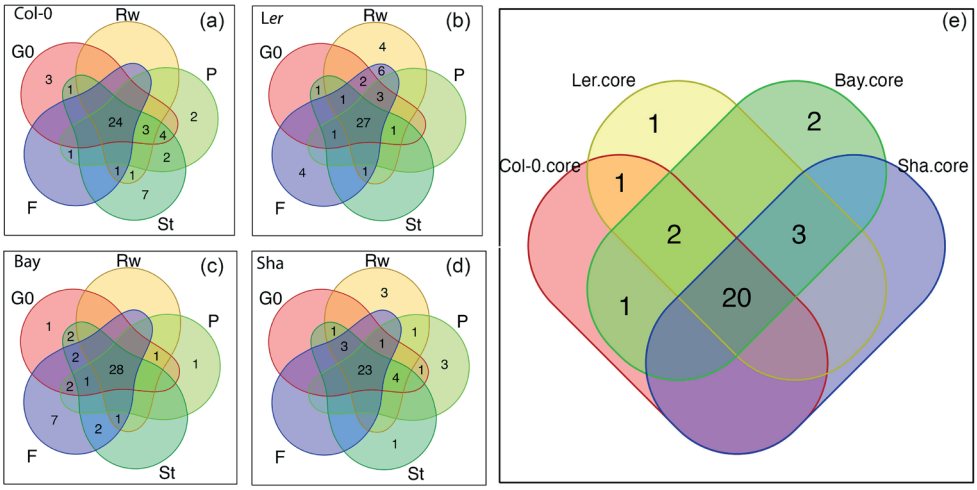


Figure 3: Shared and unique seed endophytic bacterial genera across accessions and growth substrates. Venn diagrams showing shared and unique seed endophytic bacterial taxa across accessions and growth substrates at the genus level. (a–d) Venn diagrams illustrate the overlap of bacterial genera in G0 and G3 *Arabidopsis thaliana* seeds across four growth substrates: Rockwool (Rw), Potting substrate (P), Gamma-sterilized field soil (St), and Non-sterile field soil (F), in accessions Col-0, L*er*, Bay, and Sha. (e) A conserved core microbiota comprising genera consistently present across all accessions and growth substrates.

Generational shifts in seed microbiomes depend on accession and growth substrate

To assess the influence of seed generation and growth substrate on microbial abundance, we performed differential abundance analysis between G0 and G3 seeds across all accessions and growth substrates using DESeq2. Taxa showing a significantly higher abundance in G3 relative to G0 were considered enriched, whereas taxa showing a significantly lower abundance in G3 relative to G0 were considered depleted, as indicated by the -log2 fold change values. To visualize patterns of shared and unique differentially abundant taxa across growth conditions, dot plots were generated from DESeq2 outputs, focusing on significant taxa (Fig 4; Supplementary table 6). Although qualitative analyses revealed substantial taxonomic overlap between G0 and G3 seeds, quantitative analysis revealed notable shifts in microbial abundance, with many taxa showing significant depletion in G3 seeds (Fig. 4). In Bay and Sha, G3 seeds exhibited significant depletion of multiple bacterial taxa, with no enriched (upregulated) taxa detected. In Bay, bacterial taxa such as *Bacteroidales* and *Lachnospiraceae* were notably reduced on specific substrates in G3 seeds relative to G0. Similarly, Sha seeds showed depletion of *Oscillospiraceae*, *Erysipelotrichales*, and *Lachnospiraceae* across multiple growth conditions. In contrast, Col-0 *Roseburia* was the only taxa enriched in G3 seeds on gamma-sterile substrate, while taxa such as *Azospirillum*, *Azospirillaceae*, *Blautia*, and *Parasutterella* were significantly depleted

across all substrates. L*er* displayed relatively modest shifts, with enrichment observed for *ASF356* and *Lachnospirales* and *Lactobacillales* in G3 seeds. Despite these changes, most bacterial taxa did not differ significantly between G0 and G3 seeds (Supplementary Figs. 7-10), indicating that a large portion of the seed microbiome is stably maintained across generations. These results support the existence of a conserved core microbiome, likely sustained through vertical transmission. Overall, microbial community shifts were influenced by both host genotype and growth substrate. While the core microbiome remained relatively stable across conditions, certain taxa were either lost or gained across generations and substrates.

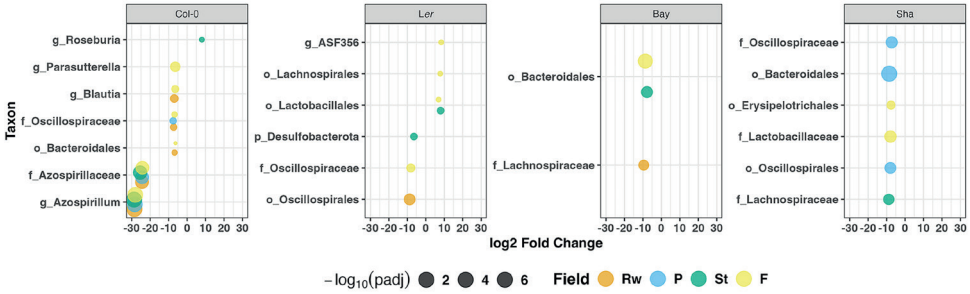


Figure 4: Differentially abundant microbial taxa between G0 and G3 seeds across *Arabidopsis thaliana* accessions and substrates. Dot plots illustrate changes in microbial abundance identified by DESeq2 analysis across four accessions. Each panel corresponds to a distinct accession. Dot colour represents the growth substrate: rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Dot size reflects statistical significance, with larger dots indicating smaller adjusted p-values. The x-axis displays $\log_2$ fold change in taxa abundance relative to G0, where positive values indicate enrichment (upregulation) and negative values indicate depletion (downregulation) in G3 seeds. The y-axis lists differentially abundant microbial taxa. Taxa were considered significant based on an adjusted p-value < 0.05 and an absolute $\log_2$(fold change) > 2.

Specific endophytic bacteria are differentially enriched in Col-0 seeds from non-sterile soils

Based on germination results, Col-0 G3 seeds from non-sterile field soil exhibited significantly higher germination rates compared to the seeds from gamma-sterilized field soil. To explore whether differences in seed endophytic communities contributed to this phenotypic variation, we performed both Venn diagram analysis and Linear Discriminant Analysis Effect Size (LEfSe) to assess community composition and differential taxonomic enrichment between the two soil treatments. Venn analysis identified a total of 44 bacterial genera, with 25 genera (56.8%) shared between both soil treatments, representing a core microbiome. Seeds from gamma-sterilized soil harbored 18 unique genera, while only one unidentified member of the family *Muribaculaceae*, was unique to seeds from non-sterile field soil (Fig. 5a; Supplementary Table 7). LEfSe analysis was

performed to identify bacterial taxa significantly associated with the two soil types. A Linear Discriminant Analysis (LDA) score threshold of 4.0 was applied to ensure high-confidence biomarker selection. A total of 8 bacterial taxa exhibited significant differences in abundance, all belonging to the phylum *Firmicutes.* Of these, five bacterial taxa, including members of the class *Clostridia*, the order *Lachnospirales*, and families *Lachnospiraceae* and *Lachnospiraceae A2*, were more abundant in seeds from gamma-sterilized field soil and were identified as biomarkers specific to this soil type. In contrast, three taxa were significantly enriched in seeds from non-sterile field soil, including the genus *Carnobacterium* (which exhibited the highest enrichment), unidentified members of the family *Carnobacteriaceae*, and bacterial taxa from the order *Lactobacillales*, serving as biomarkers for this soil type (Fig. 5b). These findings suggest that although the overall structure of the seed microbiome did not differ much between the two soil treatments (as assessed by beta diversity), specific bacterial taxa were differentially enriched. This indicates that shifts in key endophytic members may contribute to enhanced seed germination in natural, microbially rich soil.

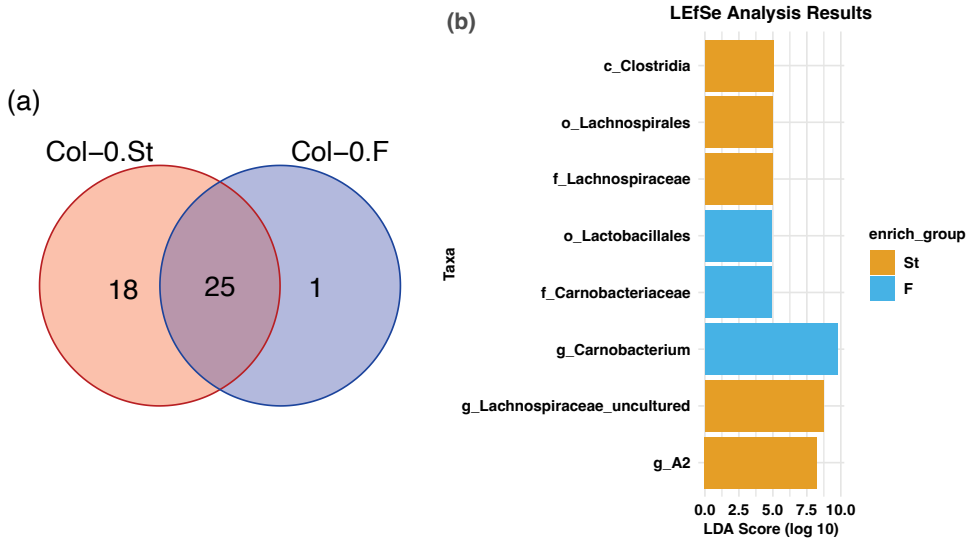


Figure 5: Differential abundance of seed endophytic bacteria in G3 Col-0 seeds from gamma-sterilized (St) and non-sterile field soil (F). (a) Venn diagram showing the number of shared and unique bacterial genera detected in seeds grown under each soil condition. (b) Histogram of Linear Discriminant Analysis (LDA) scores (>4.0, $p < 0.05$) identifying bacterial taxa with significantly different abundance between the two groups, as determined by LEfSe analysis. Taxa with longer bars indicate greater enrichment and serve as biomarkers distinguishing the seed microbiomes under the two soil treatments. Taxonomic levels are indicated as follows: c = class, o = order, f = family, g = genus.

Bacterial community composition varies across distinct substrates

To assess the impact of the growth environment on seed microbial community dynamics, we profiled the bacterial communities of four different growth substrates used over three successive generations of *Arabidopsis thaliana* cultivation. These analyses were performed on pre-planting substrate samples collected before each generation. Amplicon sequencing of microbial DNA revealed distinct bacterial community compositions across substrates. Taxonomic profiling showed that each substrate harboured a unique community, with *Bacillus* dominating both non-sterile and gamma-sterilized field soils, likely due to their common origin, while *Rhodanobacter* and *Acinetobacter* were most abundant in rockwool and potting substrates, respectively (Fig. 6a). Although gamma irradiation effectively eliminates viable microorganisms, residual DNA from non-viable cells can remain amplifiable and detectable by 16S rRNA sequencing, as observed for *Bacillus* in the sterilized field soil. These taxonomic differences were supported by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity, which revealed three major clusters of microbial communities. Non-sterile and gamma-sterilized field soils grouped closely together, while rockwool and potting substrates formed distinct and separate clusters (Fig. 6b). This pattern was further confirmed by permutational multivariate analysis of variance (PERMANOVA), which explained 58.36% of the variation in community composition (R^2 = 0.5836) and showed significant differences among substrate types (F = 20.552, p = 0.001; permutations = 999). Additionally, alpha diversity analysis revealed that non-sterile field soil supported the highest bacterial diversity, while rockwool exhibited the lowest (Fig. 6c). These substrate-specific microbial profiles likely contribute to the initial inoculum pool available for seed colonization, highlighting the importance of the growth environment in shaping the seed-associated microbiome.



Figure 6: Structure and composition of bacterial communities across four pre-planting growth substrates used over three generations of seed production. Microbial communities were characterized in rockwool (Rw), potting soil (P), gamma-sterilized field soil (St), and non-sterile field soil (F), sampled prior to planting for G1, G2, and G3 seeds from all accessions, using 16S rRNA gene amplicon sequencing. (a) Average relative abundance (%) of the thirty most dominant bacterial genera (family level shown in parentheses). Genera outside the top thirty in overall abundance were grouped under "Other" for visualization clarity. (b) Beta diversity of soil and substrate bacterial communities visualized using Non-metric Multidimensional Scaling (NMDS) based on Bray-Curtis dissimilarity. Community composition differences were tested using PERMANOVA ($p < 0.05$). (c) Alpha diversity of soil and substrate bacterial communities was measured using the Observed Diversity Index, comparing within-sample bacterial richness across substrates and generations.

Vertical transmission and environmental recruitment together shape Arabidopsis seed microbiomes

To investigate the transmission dynamics of the seed microbiome from host growth substrates across seed generations, we compared the bacterial community structures of G0 and G3 *Arabidopsis thaliana* seeds from all accessions with those of their respective growth substrates, including rockwool, potting

substrate, gamma-sterilized field soil, and non-sterile field soil. Beta diversity analysis, based on Bray-Curtis dissimilarity and visualized using non-metric multidimensional scaling (NMDS), revealed distinct clustering of seed and substrate microbiomes (Fig. 7a). The G0 and G3 seed bacterial communities clustered closely together, forming a group that was separated from the pre-planting substrate microbiota. These clustering patterns were statistically supported by permutational multivariate analysis of variance (PERMANOVA), which attributed 34.75% of the observed variation to group differences (R^2 = 0.34745; F = 64.426; P = 0.001; permutations = 999). These results suggest that a conserved seed microbiome is maintained across generations and remains distinct from the soil and substrate microbiome.

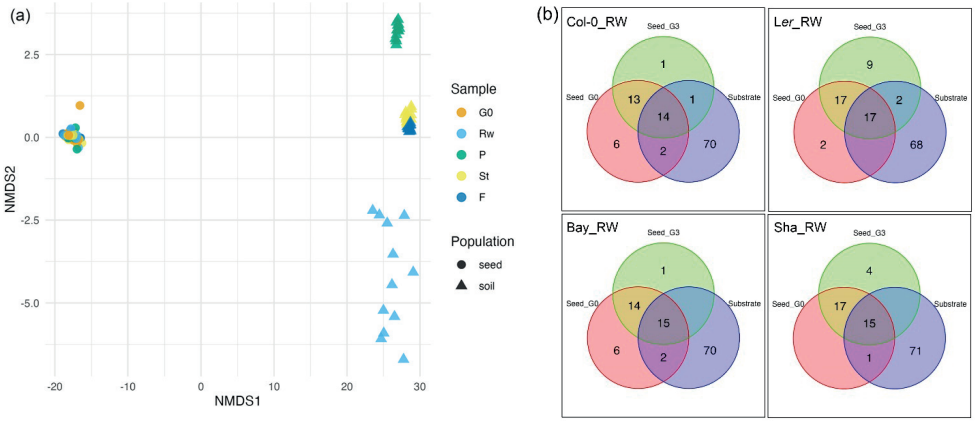


Figure 7: Bacterial community structure, beta diversity, and shared genera across seed generations and growth substrates of all Arabidopsis accessions. (a) Non-metric Multidimensional Scaling (NMDS) plot based on Bray-Curtis dissimilarity, showing the bacterial community structure of G0 and G3 *Arabidopsis thaliana* seeds and their corresponding growth substrates: rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Each point represents a biological replicate, with shapes indicating population type (seeds vs. substrates) and colors denoting sample type. Community composition differences were tested using PERMANOVA ($p < 0.05$). (b) Venn diagrams depicting shared and unique bacterial genera among G0 seeds, G3 seeds, and rockwool substrate across four accessions: Columbia-0 (Col-0), Landsberg *erecta* (Ler), Bayreuth (Bay), and Shahdara (Sha). The diagrams highlight genera that are core (shared across all three groups), as well as those unique to each seed generation or the substrate, providing insight into microbial persistence and recruitment across generations under low diversity substrate conditions.

To assess microbial recruitment during seed development, we compared bacterial genera among G0 seeds, G3 seeds from all accessions, and their respective pre-planting substrates using Venn diagram analysis. We found a small core set of bacterial genera was shared across all three sources only in seeds derived from rockwool, where it was consistently maintained across all accessions (Fig. 7b),

supporting both vertical and horizontal transmissions. Interestingly, several bacterial genera were uniquely detected only in G3 seeds but absent from both G0 seeds and pre-planting substrates, with seeds from non-sterile soil exhibited a greater number of these unique taxa (Fig. 7b; Supplementary Fig. 11). These observations suggest recruitment of seed endophytes from external sources such as air, irrigation water, or neighbouring plants during plant development. Together, these results indicate that both vertical transmission and growth substrate shape the seed microbiome.

DISCUSSION

Host environment and plant genotype critically shape seed quality, germination, and the assembly of seed-associated microbial communities (Abdelfattah et al., 2023; Vivas et al., 2015). In this study we demonstrated that both the substrate in which the mother plant grows and the genetic background of *Arabidopsis thaliana* accessions significantly shape seed germination performance under abiotic stress and determine the composition of seed endophytic bacterial communities across successive generations.

Relatively high and uniform germination was observed when seeds germinated under standard conditions across all accessions and substrates. At the same time, distinct differences became noticeable when seeds were exposed to salinity and osmotic stress (Supplementary Fig. 5). Overall, germination performance depended on both the accession and the growth substrate, with their interaction shaping the level of stress tolerance across generations. Seeds from low microbial diversity substrates, such as rockwool, consistently exhibited faster and more robust germination under stresses, likely due to continuous higher nutrient supplementation across generations, which may have mitigated stress response effects. These results suggest that nutrient availability can modulate or override microbial effects on stress-resilient seeds. These data are in agreement with a recent study by Kumari et al. (2022), which showed the importance of plant nutrition as an effective way to promote abiotic stress resilience of crops. Interestingly, the impact of the growth substrate on germination appeared to shift after three generations (by G3). We found that Col-0 seeds from non-sterile field soil displayed significantly reduced germination in early generations but had improved osmotic stress tolerance compared to those from gamma-sterilized field soil by the third generation (Fig. 1). This generational shift may reflect a progressive recruitment of selective beneficial microbiome into the seeds that enhance stress resilience, as demonstrated before in other species: tomato (Bergna et al., 2018), maize (Johnston-Monje & Raizada, 2011), rapeseed (Rochefort et al.,

2019a), wheat (Huang et al., 2016) and barley (Bziuk et al., 2021). Moreover, the enhanced stress resilience observed in G3 seeds from non-sterile soil may result from environmentally induced heritable changes, likely epigenetic modifications. For example, it was shown before that complex soil microbiota can produce compounds, such as abscisic acid like molecules or jasmonates, activating plant stress response pathways and reprogramming epigenetic marks, creating a primed state that is passed to the next generation (Doddavarapu et al., 2024; Kaya et al., 2023; Ojeilua et al., 2025). Building on these findings, future research could investigate the epigenetic mechanisms underlying the enhanced stress resilience observed in G3 seeds from non-sterile soil. For example, future studies could examine DNA methylation, histone modifications, or small RNAs (Köhler & Springer, 2017), and compare seeds from sterile and non-sterile soils to clarify how microbial signals influence heritable stress responses.

Microbiome profiling of Col-0 seeds in the third generation provides further support for the role of seed-associated bacteria in shaping germination performance under stresses. Specific bacterial taxa were differentially enriched in seeds from non-sterile soil compared to those from gamma-sterilized soil. Seeds from non-sterile field soil harboured uniquely enriched biomarker taxa, including *Carnobacterium,* and members of the order *Lactobacillales*, which were present at significantly lower abundance in seeds from gamma-sterilized soil (Fig. 5b). Interestingly, these bacteria are lactic acid bacteria, well known for their plant growth-promoting potential (Hammes & Hertel, 2006). Jain et al. (2014) reported that *Carnobacterium* strain SJ-5 from soybean produced auxins such as indole-3-acetic acid and exhibits strong 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, thereby lowering stress-induced ethylene levels in plants. Similarly, *Lactobacillus* spp., commonly found in roots and rhizospheres, was shown to enhance nutrient solubilization, synthesize phytohormones such as auxins and gibberellins, and provide biocontrol through antimicrobial compounds (Agnolucci et al., 2019; Jaffar et al., 2022). These taxa are part of the core seed microbiome we identified in this study. Still, they were found at particularly high abundance in seeds from non-sterile soil, suggesting their potential might contribute to the enhanced germination performance observed in Col-0 (Fig. 5).

Here we also showed that the effects of substrate on the seed microbial diversity were strongly genotype-dependent in agreement with other studies (Adams & Kloepper, 2002). We found that G3 Col-0 seeds produced in gamma-sterilized field soil exhibited significantly lower germination under stress conditions despite higher microbial richness. Similarly, L*er* accession showed relatively high seed microbial richness when grown in rockwool. However, for Bay and Sha seeds, no substrate effect on microbial diversity was observed (Fig. 2b). These

findings indicate that the influence of the growth substrate on seed microbiome assembly varies among host genotypes and suggest that host genetic background plays an important role in shaping seed-associated microbial communities. In contrast to previous studies suggesting positive effects of microbial diversity on plant performance (Ugarte et al., 2024; Walsh et al., 2021), our results did not show a consistent positive relationship between seed microbial richness and seed performance in this system. Higher microbial richness was not always associated with improved germination or stress tolerance across the different genotypes and growth substrates. Because differences in microbial richness were accompanied by differences in plant genotype and growth substrate, we cannot conclude that microbial richness alone explains the observed differences in seed performance. Instead, these findings suggest that the composition of seed-associated microbial communities may be more important than microbial richness itself in determining plant performance under stress conditions.

Collectively, our amplicon sequencing analyses revealed that Arabidopsis seeds harbour a conserved core microbiome that dominates the bacterial community and persists across accessions, substrates, and generations. The Arabidopsis core seed microbiome consisted of 20 bacterial taxa, primarily represented by members of Firmicutes (55%) and Bacteroidota (35%), with minor contributions from Deferribacterota (5%) and Campylobacterota (5%) (Supplementary table 5). However, this taxonomic composition differs markedly from previously described global seed core microbiomes. For example, the global seed core identified across 50 plant species by Simonin et al. (2022) consisted of 13 bacterial taxa dominated by Proteobacteria (84.6%), mainly represented by *Pantoea* and *Pseudomonas*, with smaller contributions from Actinobacteriota (unclassified *Microbacteriaceae*, 7.6%) and Firmicutes (*Paenibacillus*, 7.6%). Similarly, (Johnston-Monje et al., 2021), in their analysis of seeds core microbiome from 17 plant species, reported that 15 bacterial operational taxonomic units (OTUs) were detected in more than 60% of species, but only *Pantoea* and *Enterobacter,* both belonging to the phylum Proteobacteria, were core members present across all species. Moreover, our Arabidopsis core microbiome included several taxa not reported as members of the global seed core, including *Lachnospiraceae*, *Lactobacillaceae*, *Muribaculaceae*, *Rikenellaceae*, *Oscillospiraceae*, *Mucispirillum*, *Prevotellaceae*, *Deferribacteraceae*, and *Erysipelotrichaceae*.

Interestingly, many of these taxa are more commonly associated with animal gut microbiomes (Song et al., 2024; Yang et al., 2021). However, several members have previously been reported in seed-associated microbial communities of other plant species. Members of the family *Lachnospiraceae* were identified in the seed microbiome of *Brassica parachinensis* (Wang et al., 2025), while members of the

Lactobacillaceae were isolated from seeds of multiple cucurbit species (Khalaf & Raizada, 2016). Similarly, *Prevotellaceae*, *Deferribacteraceae*, *Erysipelotrichaceae*, *Lachnospiraceae*, and *Lactobacillaceae* were detected in seedlings grown from surface-sterilized Miscanthus seeds, suggesting vertical transmission of these taxa through seeds (Cope-Selby et al., 2017). The occurrence of several gut-associated taxa that are rarely reported from plant seeds may reflect limitations in taxonomic assignment based on 16S rRNA gene sequences, contamination introduced during sample processing, or the presence of seed-associated microorganisms that have been overlooked in previous seed microbiome studies. Further validation using cultivation, microscopy, or genome-based approaches would be needed to confirm whether they are indeed microorganisms residing in the seeds. Overall, the persistence of these core taxa from G0 to G3 supports vertical transmission as an important mechanism underlying the maintenance of seed-associated microorganisms across generations.

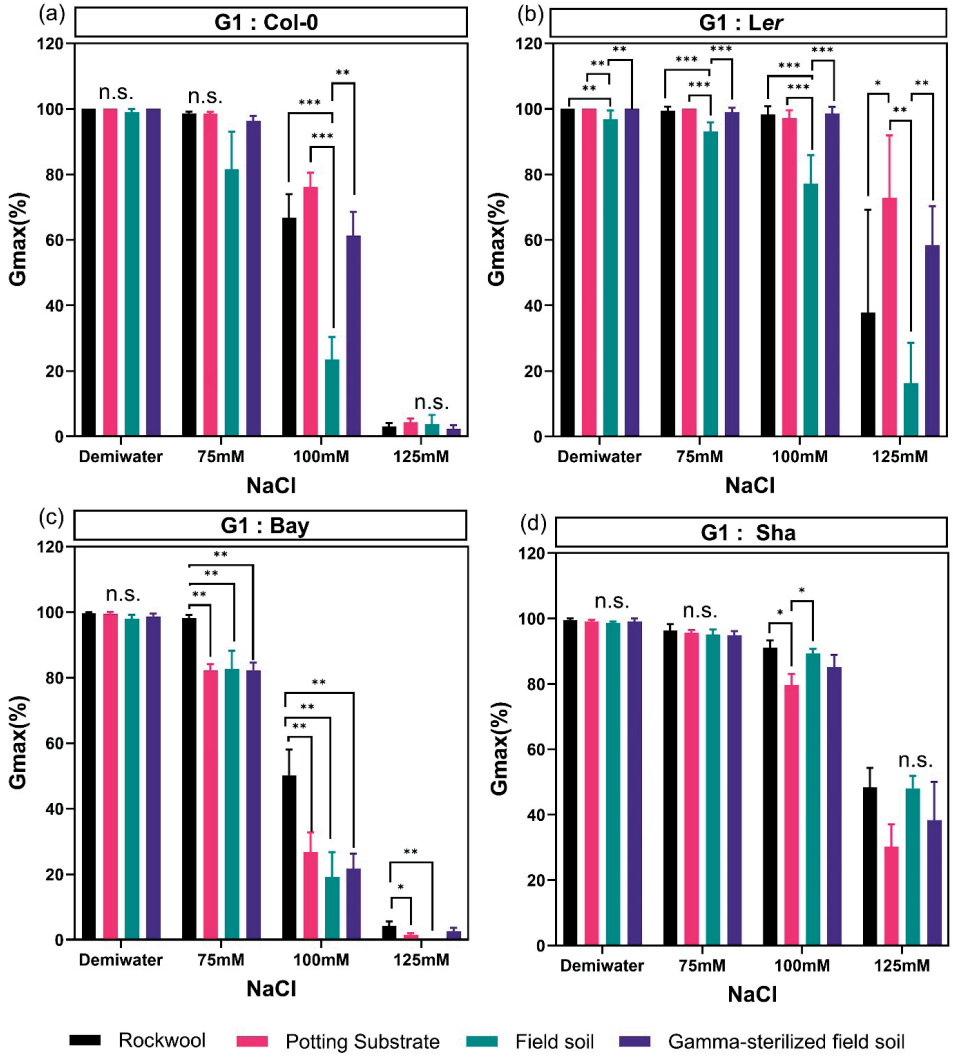
The vertical transmission of seed bacterial endophytes has been documented in numerous plant species, including barley, rice, and bean, with many core members exhibiting plant beneficial traits (Bziuk et al., 2021; Sulesky-Grieb et al., 2024; Zhang et al., 2022). These core bacteria enhance plant growth, health, and stress resilience. For instance, in rice, *Flavobacterium* sp. strains from seeds solubilize phosphate and produce indole-3-acetic acid, enhancing nutrient availability and improving tolerance to abiotic stresses such as salinity and drought (Walitang et al., 2017). In *Salvia miltiorrhiza*, core seed endophytes support secondary metabolite production and stress tolerance (Chen et al., 2018), while in rice, they also provide biocontrol against pathogens, boosting seedling vigour under abiotic stress (Jana et al., 2023; Rana & Patil, 2024). Consistent with this, beta diversity analyses in our study confirmed that overall community composition remained largely stable across substrates and accessions, reinforcing the persistence of this host-selected core seed microbiome. However, many taxa showed significant depletion in G3 seeds across all substrates and accessions, suggesting that vertical transmission is subjected to a bottleneck effect, where some microbial taxa are unable to be transmitted to the seeds of the next generation, especially under microbe-limited greenhouse conditions (Newcombe et al., 2018; Rodríguez et al., 2020). This bottleneck restriction has been reported in radish, where seed microbial communities showed low heritability and variation across plant generations due to dramatic ecological drift (Rezki et al., 2018). Furthermore, Abdelfattah et al. (2023) reported that the transition from seed to seedling represents a bottleneck, as seeds typically harbour higher microbial diversity than seedlings, indicating that many inherited microorganisms fail to establish in the developing seedling and thus reduce the probability of colonizing the next generation of plants.

In addition to the vertically transmitted core microbiome, our results highlight a role for horizontal transmission in shaping a small fraction of the seed bacterial community (Fig. 7; Supplementary 11). This observation is supported by the study of Zhou et al. (2020), which showed that inheritance from parental seeds outweighed recruitment from the rhizosphere, with values of 25.5 percent and 10.7 percent, respectively. Several bacterial genera were detected exclusively in G3 seeds but absent from both G0 seeds and the pre-planting substrates, with seeds from non-sterile soil showing the highest number of these unique taxa. Because soil hosts an extremely diverse microbial community, niche seed-associated taxa present at low abundance are easily masked, even with deep sequencing, thereby reducing the apparent overlap between seed and soil microbiomes (Alteio et al., 2020). Another possible explanation is that, although a PNA clamp was applied to reduce amplification of plant DNA during 16S rRNA sequencing, residual host sequences may have reduced the effective microbial sequencing depth, potentially limiting detection of extremely low-abundance taxa in seed samples. Hussain et al. (2025) found that PNA clamps markedly reduce, but did not completely remove, plant chloroplast and mitochondrial DNA during PCR. Likewise, Viotti et al. (2024) showed that PNA clamps increase the proportion of fungal reads but have little effect on overall diversity or community composition at higher sequencing depth. These observations together suggest that the unique, horizontally acquired taxa identified in G3 seeds were both rare and may have been acquired from external sources, possibly from neighbouring plants (e.g. mosses that colonized the non-sterile soil surface), irrigation water, or airborne deposition during seed development (Santoyo, 2022; Wang & Zhang, 2023). In contrast, only G3 seeds grown on rockwool exhibited shared core microbial taxa with both the substrate and G0 seeds. This may be because the G0 seeds were grown on rockwool for many cycles before the start of this study, and then continued on rockwool for three additional generations. It was shown before that the seed microbiome may require many generations for soil microbes to be recruited through the roots, translocated via the plant xylem, and integrated into the seed microbial community (Abdelfattah et al., 2021; Russell et al., 2025). Another possible reason that we did not detect broader transmission from substrates/soils to seeds may be related to our sampling strategy. We collected pre-growth substrate/soil samples, which represent bulk soil, rather than sampling the rhizosphere or substrate near the root zone during plant growth. Since the rhizosphere is known to harbour the highest microbial abundance and is the main site of plant-driven microbial recruitment, the lack of rhizosphere sampling may have limited our ability to capture potential microbial transmission pathways from soil/substrate to seeds, as suggested also in other studies (Chamkhi et al., 2022; Chaudhary et al., 2015). Additionally, controlled greenhouse conditions likely reduce environmental exposure and selection pressures, limiting opportunities for

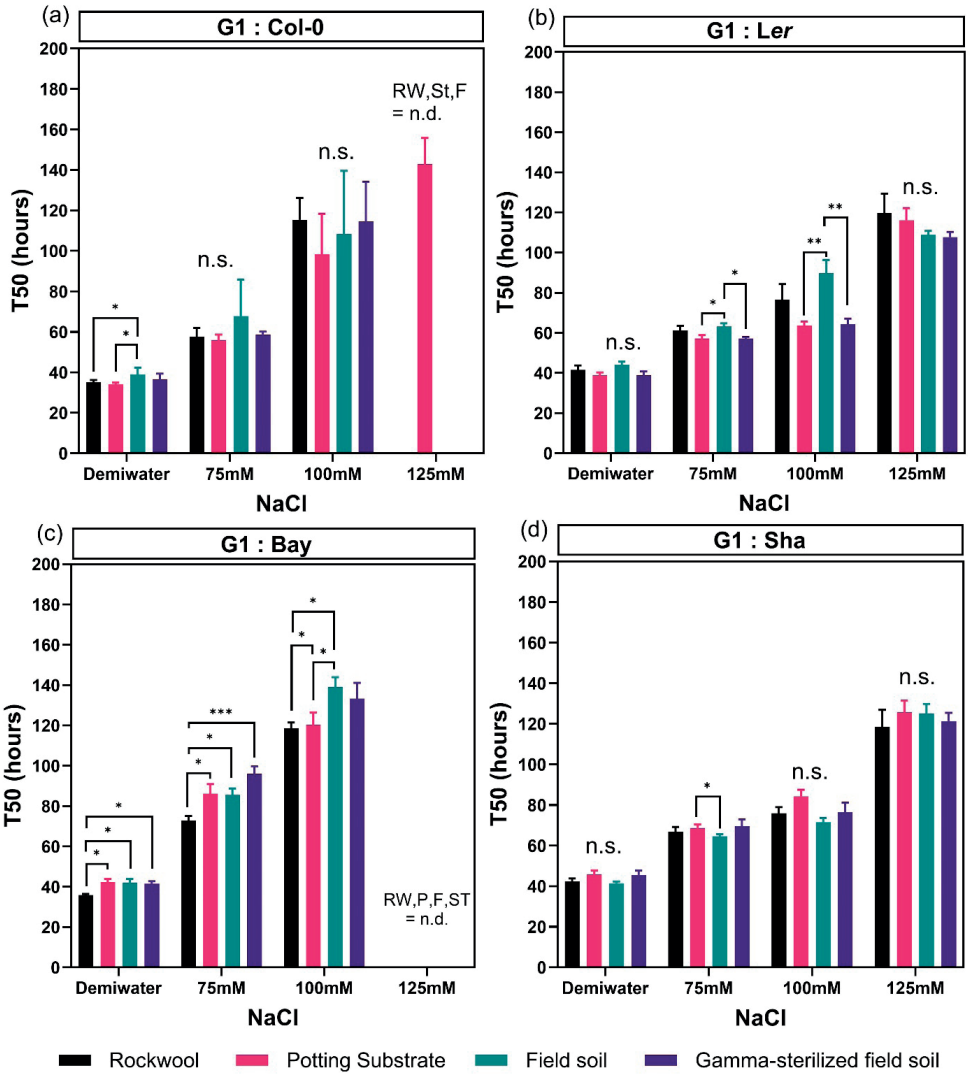
endophyte colonization and resulting in a stable but less diverse seed microbiome compared to plants grown under variable field conditions or stresses (Bintarti et al., 2022; Sharon et al., 2023). Seeds produced in the field generally exhibit higher and more complex endophytic bacterial diversity due to critical exposure to soil and environmental microbial reservoirs, which serve as key sources for seed microbiota. In contrast, greenhouse or sterile conditions reduce external microbial input, causing seed endophytic communities to decline in diversity over successive generations, with composition becoming less complex and dominated by a few taxa (Bergna et al., 2018; Rodríguez et al., 2020; Walitang et al., 2019).

In conclusion, our study demonstrates that Arabidopsis seed endophytic bacteria and germination are influenced by both host genotype and growth substrate. We identify the composition of the Arabidopsis seed core endophytes and show that seed microbiomes are largely shaped by a vertically inherited, stable core community conserved across accessions, substrates, and generations, alongside a smaller, more variable fraction influenced by horizontal transmission, substrate-specific factors, and environmental inputs. Evidence of direct transmission from substrate to seeds was observed only in seeds continuously grown on rockwool for extended periods, suggesting that prolonged cultivation may be required for soil microbes to integrate into seeds. Germination assays indicate that seed-associated microbes can enhance stress resilience, with the enrichment of lactic acid bacteria beneficial taxa in seeds from non-sterile soils potentially promoting seed germination under stress. Future studies should integrate the isolation of core bacterial endophytes with transcriptome profiling and microbiome functional analyses to resolve the molecular pathways linking microbes and the inherited core seed microbiome to seed physiology and survival (stress resilience). This knowledge can guide seed treatments and breeding programs to select plants or growth substrates/conditions that attract beneficial microbes or drive the seed microbiome to a desired composition for specific field conditions, offering a sustainable way to enhance seed vigour and stress tolerance. Using this knowledge obtained from plant-microbe interactions could lead to improving crop performance and resilience and support global food security in the future.

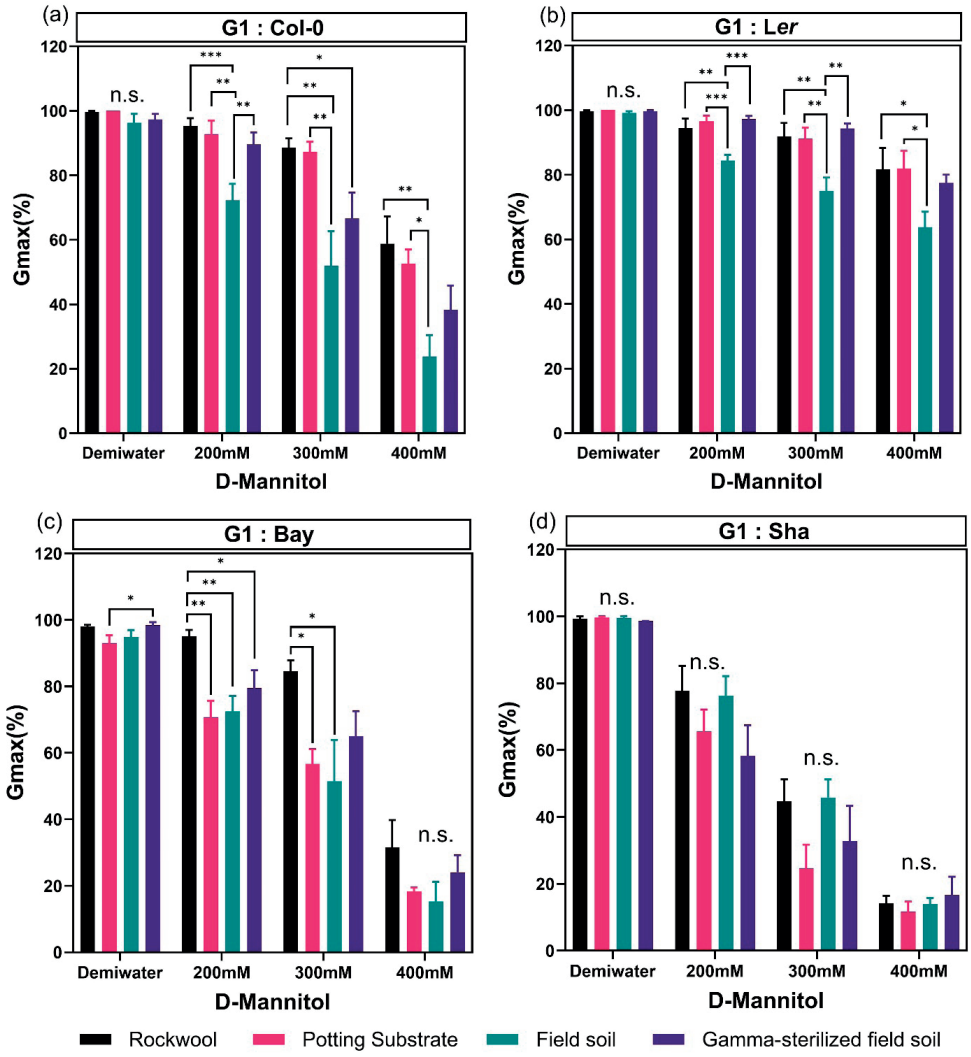
SUPPLEMENTARY FIGURES



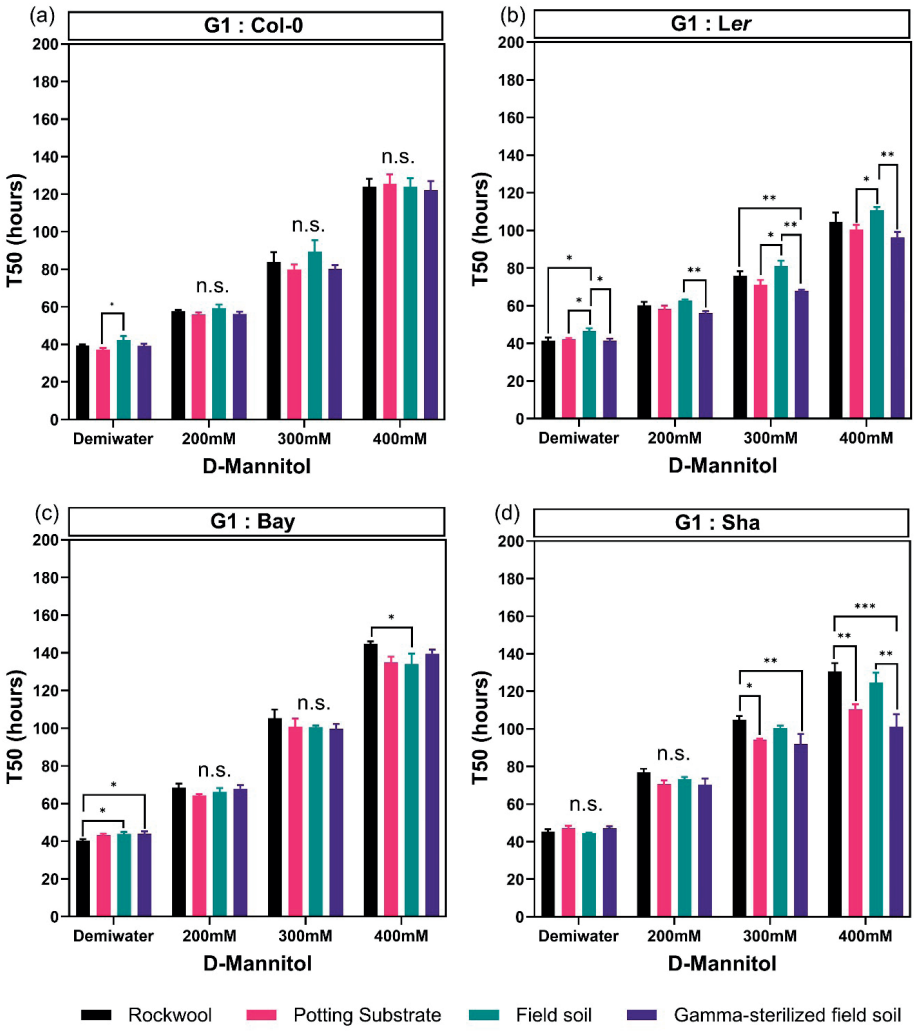
Supplementary Figure 1: Germination percentage of G1 *Arabidopsis thaliana* seeds under salt stress across accessions and growth substrates. Average germination percentage (Gmax) of G1 *A. thaliana* seeds from four accessions: (a) Columbia-0 (Col-0), (b) Landsberg *erecta* (Ler), (c) Bayreuth (Bay), and (d) Shahdara (Sha), cultivated on four different growth substrates: rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Germination was assessed under control conditions (demineralized water) and under salt stress induced by 75 mM, 100 mM, and 125 mM NaCl. Data represent means ± standard error (s.e.) from four biological replicates. Statistical significance was assessed using one-way ANOVA followed by LSD post hoc test. Significance is indicated as follows: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



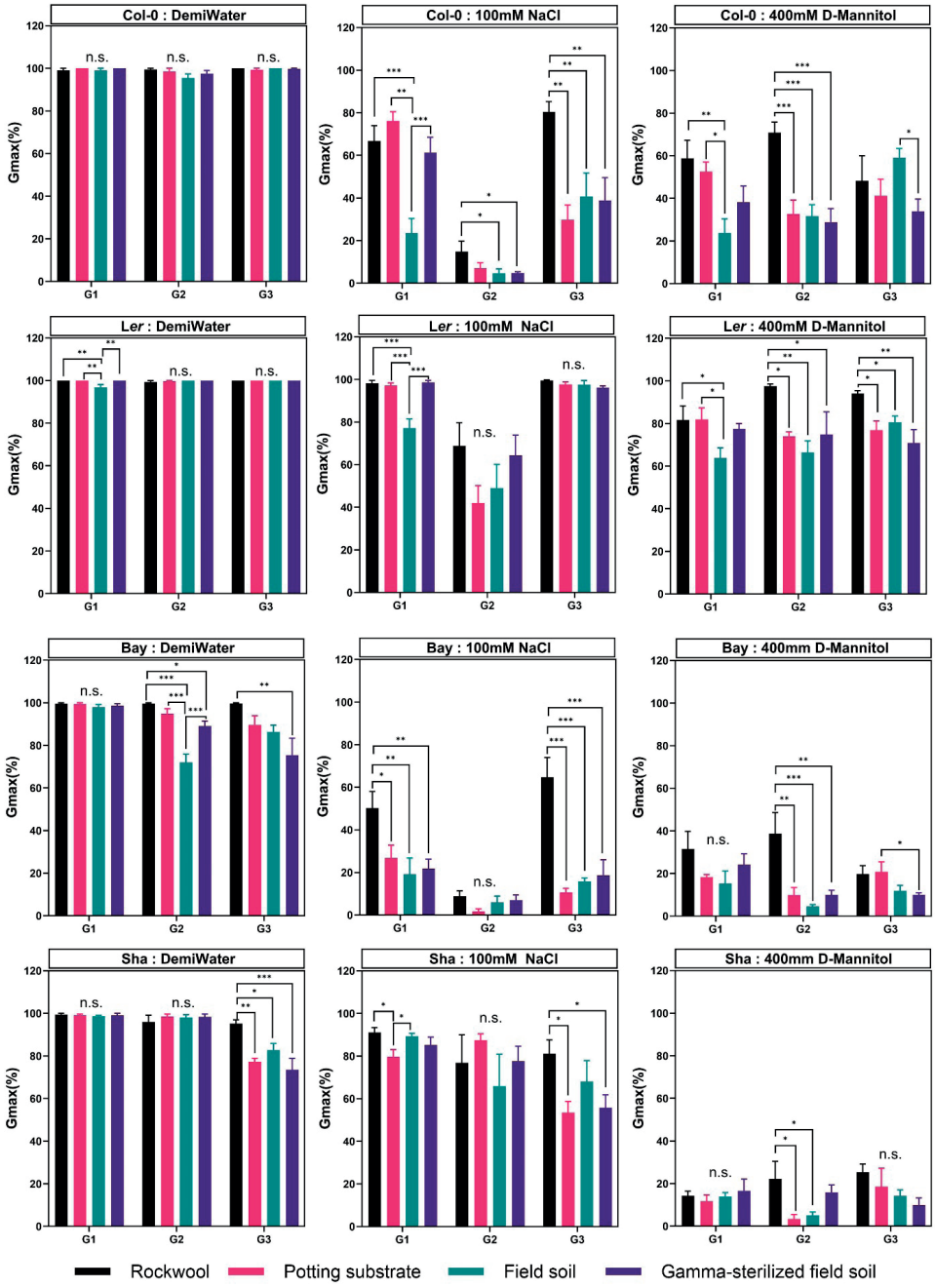
Supplementary Figure 2: Germination rate (T50) of G1 *Arabidopsis thaliana* seeds across accessions and growth substrates under salt stress. Mean time to reach 50% of total germination (T50) of G1 seeds from four A. thaliana accessions: (a) Columbia-0 (Col-0), (b) Landsberg *erecta* (L*er*), (c) Bayreuth (Bay), and (d) Shahdara (Sha), cultivated on four substrates: rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Seeds were germinated under control conditions (demineralized water) and under salt stress induced by 75 mM, 100 mM, and 125 mM NaCl. Data represent mean ± s.e. from four biological replicates. Statistical significance was determined by one-way ANOVA followed by LSD post hoc test. n.d., fewer than three replicates produced valid T50 estimates; n.s., not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



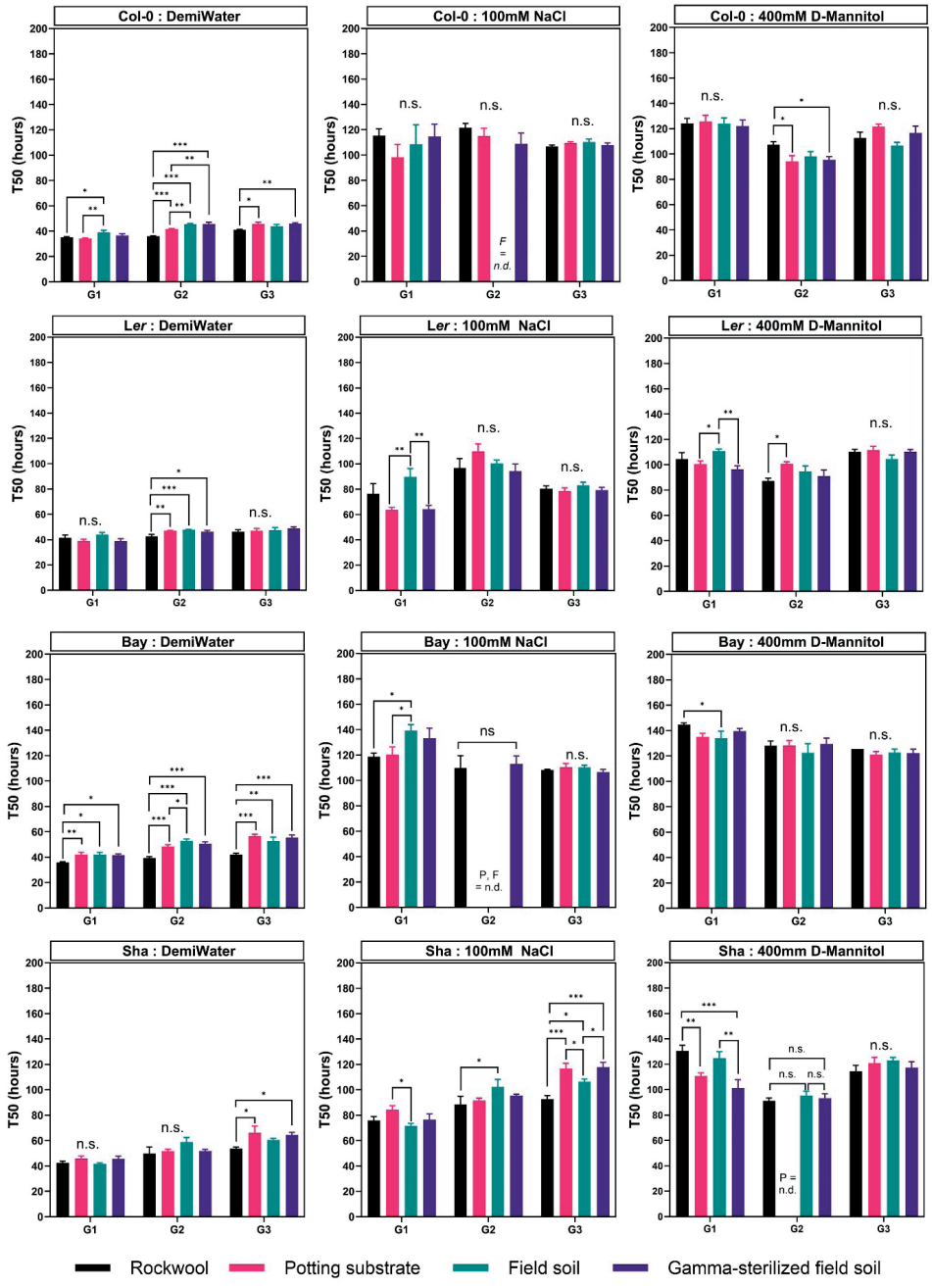
Supplementary Figure 3: Germination percentage of G1 *Arabidopsis thaliana* seeds under osmotic stress across accessions and growth substrates. Average germination percentage (Gmax) of G1 *A. thaliana* seeds from four accessions: (a) Columbia-0 (Col-0), (b) Landsberg *erecta* (Ler), (c) Bayreuth (Bay), and (d) Shahdara (Sha), cultivated on four different growth substrates: rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Germination was assessed under control conditions (demineralized water) and under osmotic stress induced by 200 mM, 300 mM, and 400 mM D-Mannitol. Data represent means ± standard error (s.e.) from four biological replicates. Statistical significance was assessed using one-way ANOVA followed by LSD post hoc test. Significance is indicated as follows: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



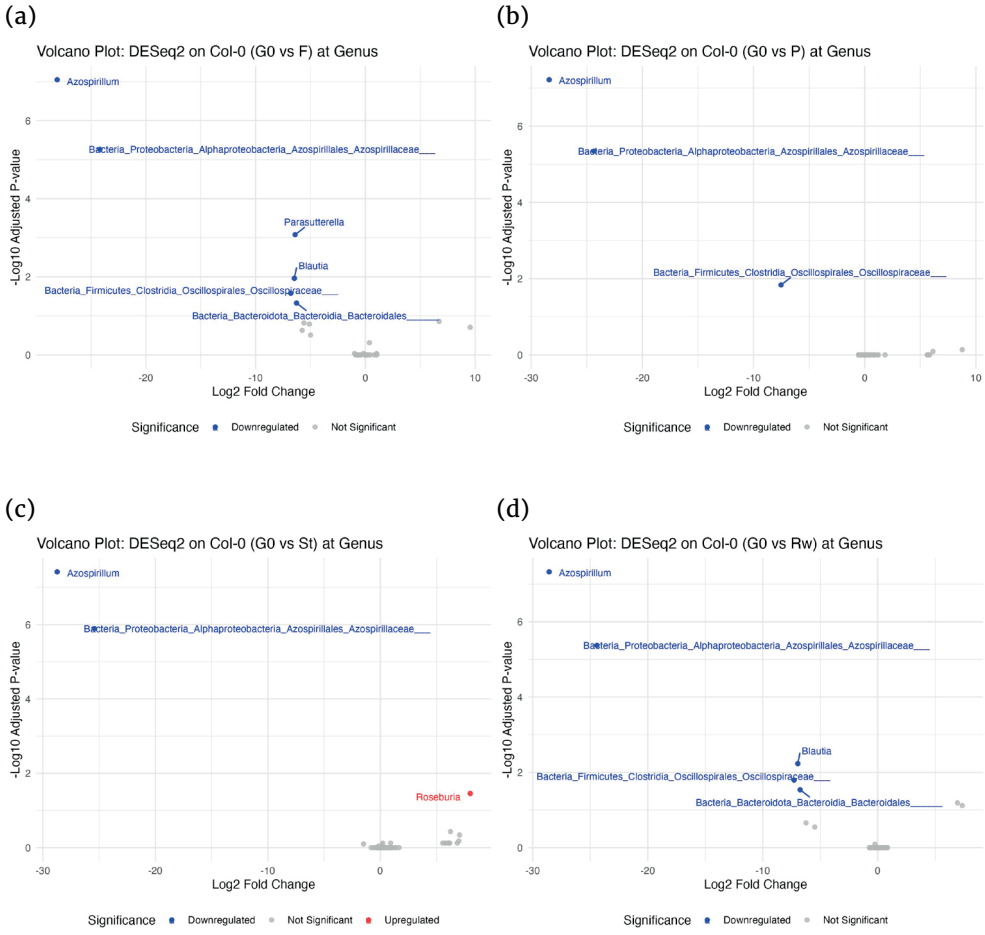
Supplementary Figure 4: Germination rate (T50) of G1 *Arabidopsis thaliana* seeds across accessions and growth substrates under salt stress. Mean time to reach 50% of total germination (T50) of G1 seeds from four A. thaliana accessions: (a) Columbia-0 (Col-0), (b) Landsberg *erecta* (L*er*), (c) Bayreuth (Bay), and (d) Shahdara (Sha), cultivated on four substrates: rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Seeds were germinated under control conditions (demineralized water) and under osmotic stress induced by 200 mM, 300 mM, and 400 mM D-Mannitol. Data represent mean ± s.e. from four biological replicates. Statistical significance was determined by one-way ANOVA followed by LSD post hoc test. n.d., fewer than three replicates produced valid T50 estimates; n.s., not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.


Col-0 : DemiWater
Col-0 : 100mM NaCl
Col-0 : 400mM D-Mannitol
Ler : DemiWater
Ler : 100mM NaCl
Ler : 400mM D-Mannitol
Bay : DemiWater
Bay : 100mM NaCl
Bay : 400mm D-Mannitol
Sha : DemiWater
Sha : 100mM NaCl
Sha : 400mm D-Mannitol
Gmax(%)
G1
G2
G3
n.s.
Rockwool
Potting substrate
Field soil
Gamma-sterilized field soil

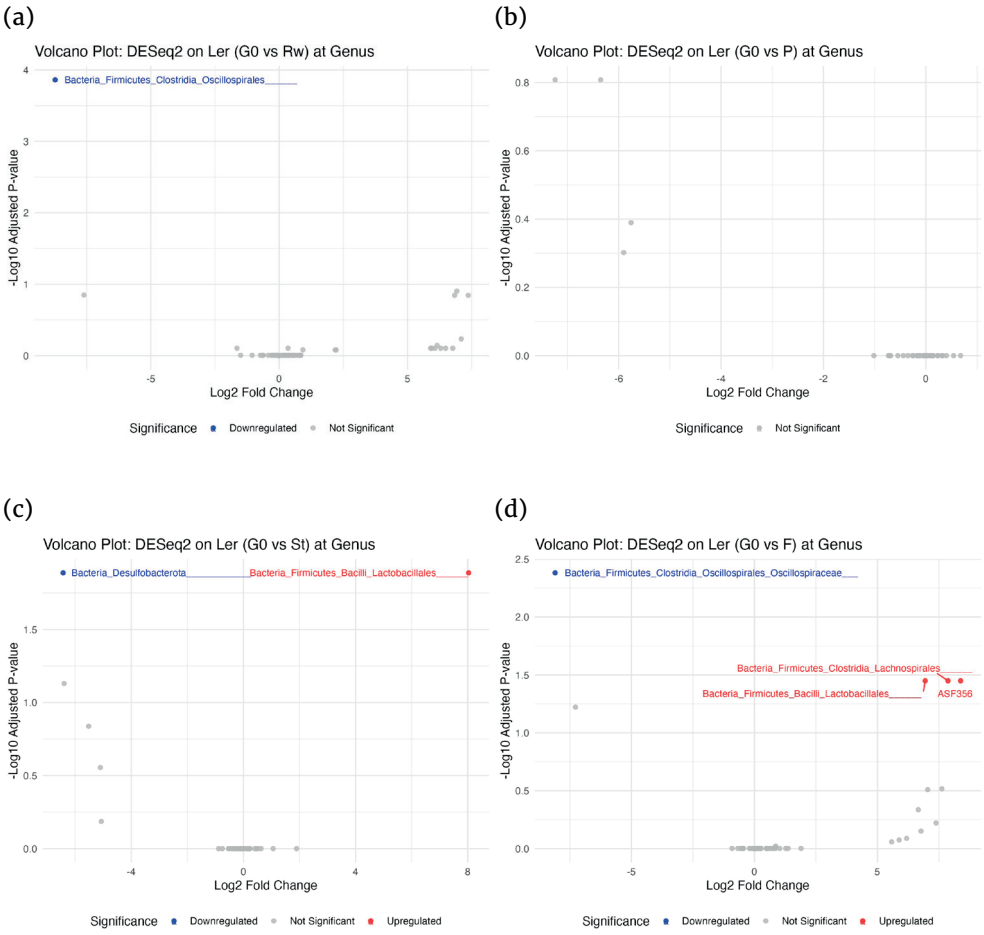
Supplementary Figure 5: Germination percentage of G1-G3 *Arabidopsis thaliana* seeds across accessions, substrates and stress conditions. Average germination percentage of G1-G3 seeds from four A. thaliana accessions: Columbia-0 (Col-0), Landsberg erecta (Ler), Bayreuth (Bay), and Shahdara (Sha), cultivated on four growth substrates: rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Germination was assessed under control conditions (demineralized water), salinity stress (100 mM NaCl), and osmotic stress (400 mM D-mannitol). Data represent means ± standard error (s.e.) from four biological replicates. Statistical significance was assessed using one-way ANOVA followed by LSD post hoc test. Significance is indicated as follows: ns, not significant ($P > 0.05$); * significant, ($P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Panels: (a-c) Col-0 under control, salinity, and osmotic stress; (d-f) Ler; (g-i) Bay, and (j-l) Sha, respectively.


Col-0 : DemiWater
Col-0 : 100mM NaCl
Col-0 : 400mM D-Mannitol
Ler : DemiWater
Ler : 100mM NaCl
Ler : 400mM D-Mannitol
Bay : DemiWater
Bay : 100mM NaCl
Bay : 400mm D-Mannitol
Sha : DemiWater
Sha : 100mM NaCl
Sha : 400mm D-Mannitol
T50 (hours)
G1
G2
G3
n.s.
ns
F = n.d.
P, F = n.d.
P = n.d.
Rockwool
Potting substrate
Field soil
Gamma-sterilized field soil

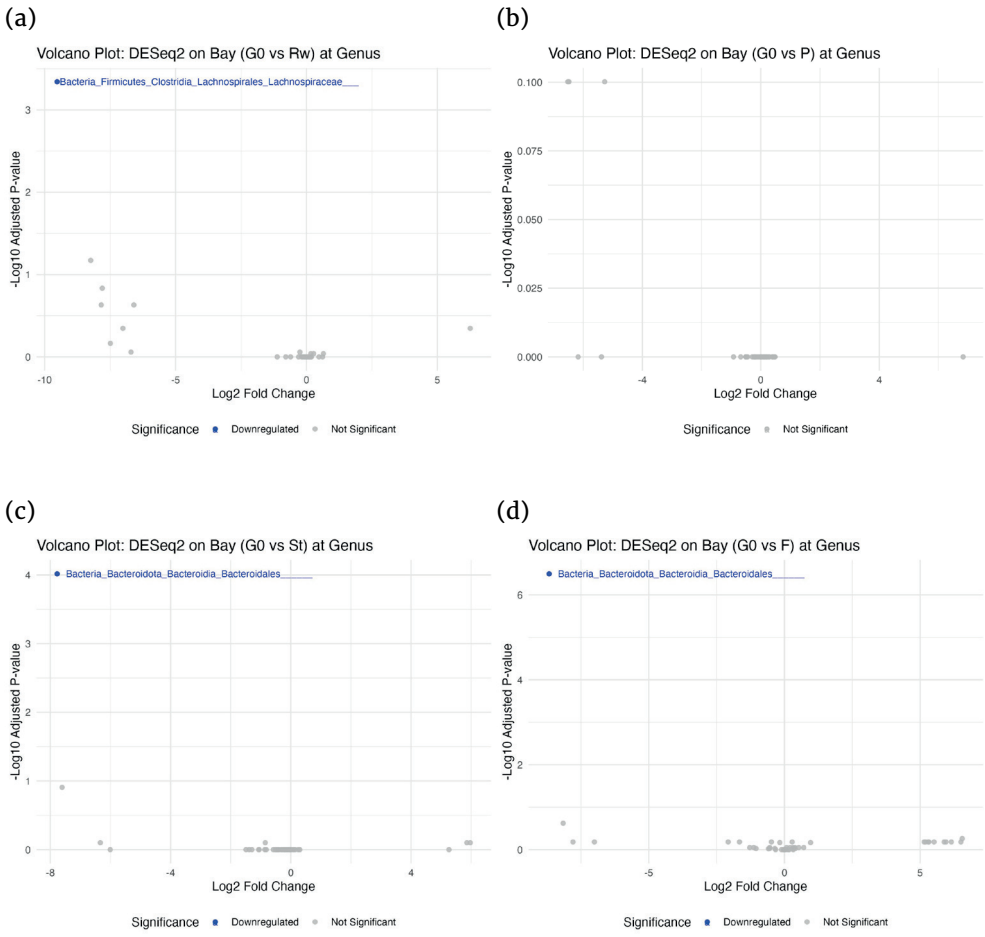
Supplementary Figure 6: Germination speed (T50) of G1-G3 *Arabidopsis thaliana* seeds across accessions, substrates, and stress conditions. Average T50 (time to reach 50% of total germination) of G1- G3 *A. thaliana* seeds from four accessions: Columbia-0 (Col-0), Landsberg *erecta* (L*er*), Bayreuth (Bay), and Shahdara (Sha), cultivated on four different growth substrates: rockwool, potting substrate, gamma-sterilized field soil, and non-sterile field soil. Seeds were germinated under control (demineralized water), salinity stress (100 mM NaCl), and osmotic stress (400 mM D-mannitol). Data represent mean ± s.e. from four biological replicates. Statistical significance was determined by one-way ANOVA followed by LSD post hoc test. n.d., fewer than three replicates produced valid T50 estimates; n.s., not significant ($P > 0.05$); * significant, ($P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Panels: (a-c) Col-0 under control, salinity, and osmotic stress; (d-f) L*er*; (g-i) Bay, and (j-l) Sha, respectively.



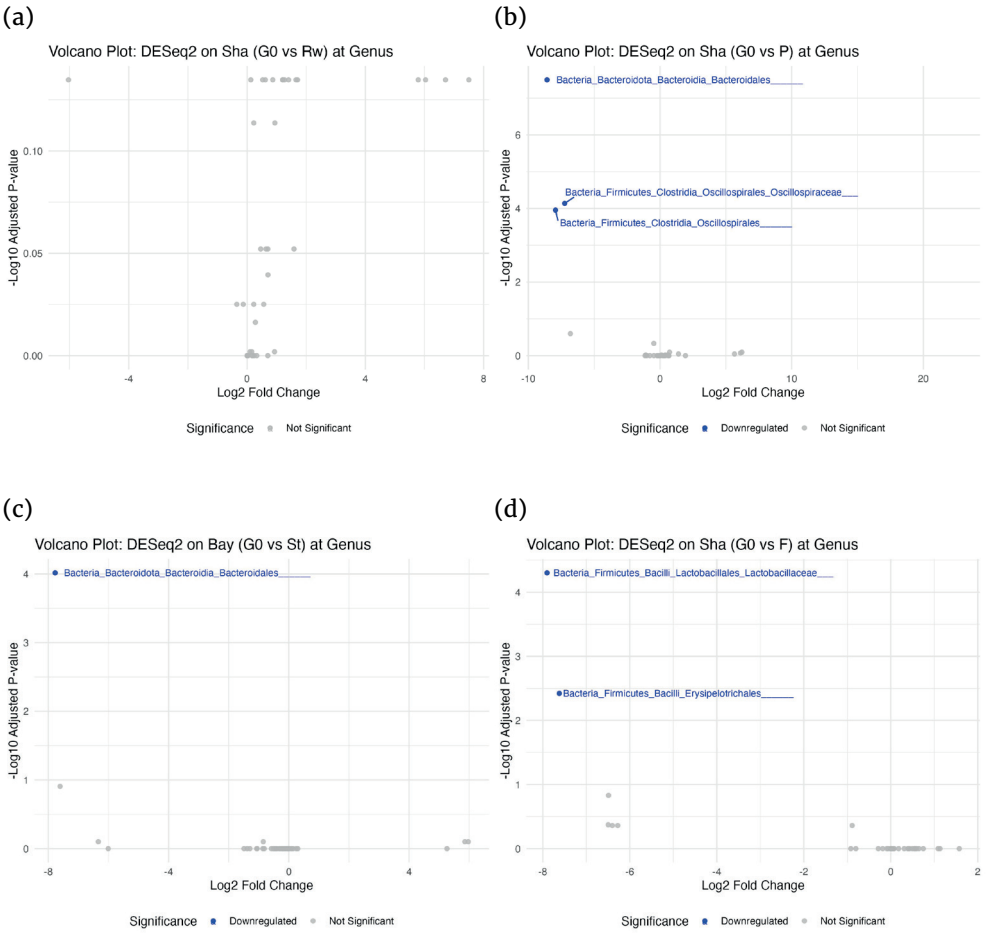
Supplementary Figure 7: Differentially abundant seed endophytic bacterial genera between G0 and G3 *Arabidopsis thaliana* Columbia-0 (Col-0) seeds across four growth substrates. Volcano plots showing differential abundance of seed endophytic bacterial genera between G0 and G3 seeds from (a) rockwool (Rw), (b) potting substrate (P), (c) gamma-sterilized field soil (St), and (d) non-sterile field soil (F). Differential abundance analysis was performed using DESeq2. Genera with an adjusted $P < 0.05$ and absolute $\log_2$(fold change) > 2 were considered significantly different. Red dots represent taxa enriched in G3 seeds (upregulated), blue dots indicate enrichment in G0 seeds/depleted in G3 (downregulated), and grey dots represent non-significant taxa.



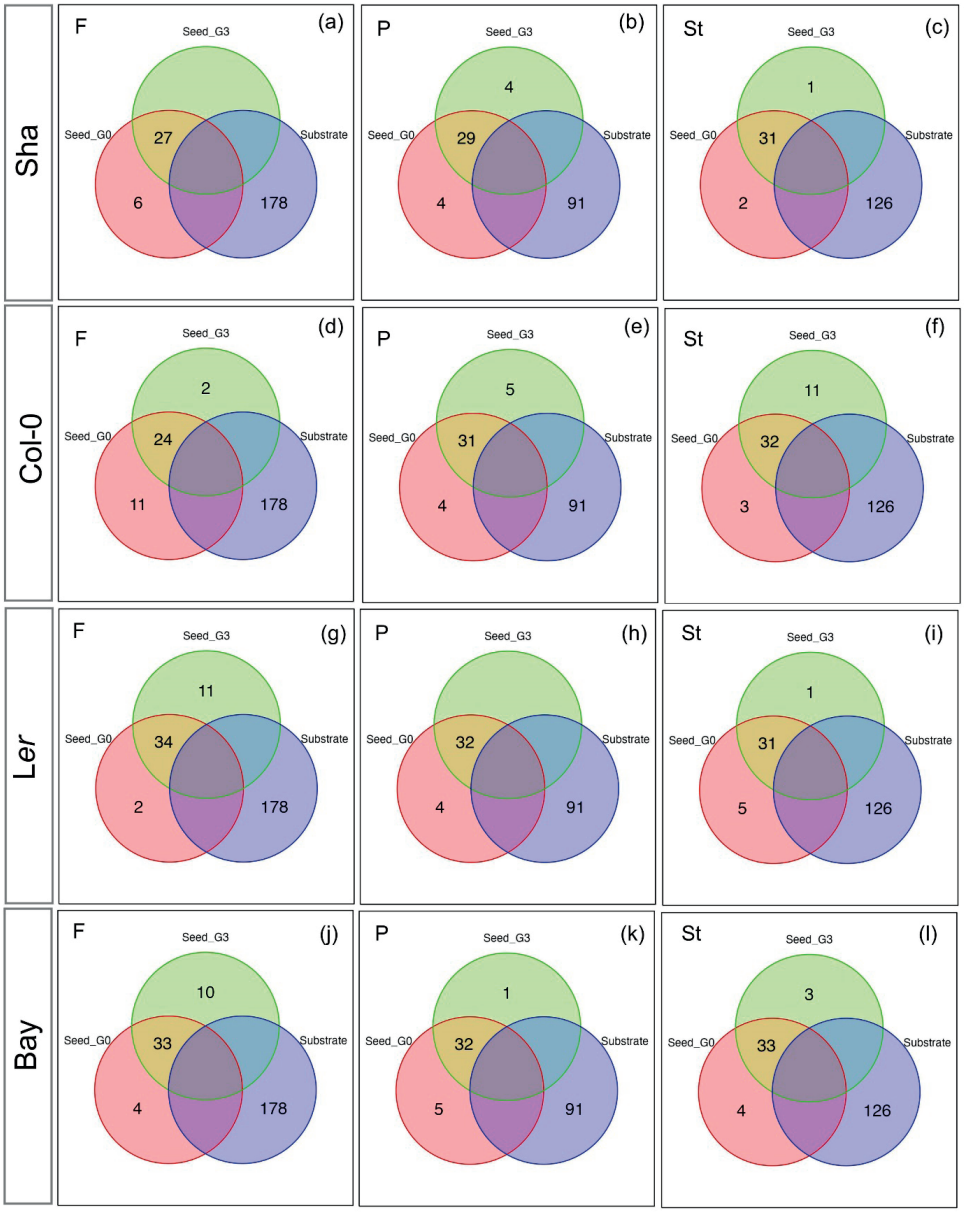
Supplementary Figure 8 : Differentially abundant seed endophytic bacterial genera between G0 and G3 *Arabidopsis thaliana* Landsberg *erecta* (L*er*) seeds across four growth substrates. Volcano plots showing differentially abundant seed endophytic bacterial genera comparing between G0 and G3 seeds from (a) rockwool (Rw), (b) potting substrate (P), (c) gamma-sterilized field soil (St), and (d) non-sterile field soil (F). Differential abundance analysis was performed using DESeq2. Genera with an adjusted $P < 0.05$ and absolute $\log_2$(fold change) > 2 were considered significantly different. Red dots represent taxa enriched in G3 seeds (upregulated), blue dots indicate enrichment in G0 seeds/depleted in G3 (downregulated), and grey dots represent non-significant taxa.



Supplementary Figure 9: Differentially abundant seed endophytic bacterial genera between G0 and G3 *Arabidopsis thaliana* Bayreuth (Bay) seeds across four growth substrates. Volcano plots showing differentially abundant seed endophytic bacterial genera comparing between G0 and G3 seeds from (a) rockwool (Rw), (b) potting substrate (P), (c) gamma-sterilized field soil (St), and (d) non-sterile field soil (F). Differential abundance analysis was performed using DESeq2. Genera with an adjusted $P < 0.05$ and absolute $\log_2$(fold change) > 2 were considered significantly different. Red dots represent taxa enriched in G3 seeds (upregulated), blue dots indicate enrichment in G0 seeds/depleted in G3 (downregulated), and grey dots represent non-significant taxa.



Supplementary Figure 10: Differentially abundant seed endophytic bacterial genera between G0 and G3 *Arabidopsis thaliana* Shahdara (Sha) seeds across four growth substrates. Volcano plots showing differentially abundant seed endophytic bacterial genera comparing between G0 and G3 seeds from (a) rockwool (Rw), (b) potting substrate (P), (c) gamma-sterilized field soil (St), and (d) non-sterile field soil (F). Differential abundance analysis was performed using DESeq2. Genera with an adjusted $P < 0.05$ and absolute $\log_2$(fold change) > 2 were considered significantly different. Red dots represent taxa enriched in G3 seeds (upregulated), blue dots indicate enrichment in G0 seeds/depleted in G3 (downregulated), and grey dots represent non-significant taxa.



Supplementary Figure 11: Shared and unique bacterial genera among G0 seeds, G3seeds, and soil/substrate types across Arabidopsis accessions. Venn diagrams showing the overlap of endophytic bacterial genera detected in G0 seeds, G3 seeds, and associated substrates including potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F) for four *Arabidopsis thaliana* accessions: Shahdara (Sha; a-c), Columbia-0 (Col-0; d-f), Landsberg *erecta* (Ler; g-i), and Bayreuth (Bay; j-l).

SUPPLEMENTARY TABLES

Supplementary Table 1: Taxonomic classification of shared and unique seed endophytic bacterial genera in G0 and G3 *Arabidopsis thaliana* Columbia-0 (Col-0) seeds across four substrates. Taxonomic classification of shared and unique seed endophytic bacteria detected in Col-0 accession of G0 and G3 cultivated on four different growth substrates: rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Bacterial taxa are presented at five taxonomic ranks: phylum, class, order, family, and genus, based on 16S rRNA gene amplicon sequencing.

Shared/Unique	Phylum	Class	Order	Family	Genus
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*uncultured_bacterium*
core	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
core	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured*
core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
core	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*uncultured*
core	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_NK4A136 _group*
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
core	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*
core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*

Supplementary Table 1: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*
core	Firmicutes	*Bacilli*	*Erysipelotrichales*	-	-
core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	-
core	Firmicutes	*Clostridia*	*Oscillospirales*	-	-
core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	-
Rw_St_F	Deferribacterota	*Deferribacteres*	*Deferribacterales*	-	-
P_F	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	-
Go_Rw_P_St	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
Go_Rw_P_St	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
Go_Rw_P_St	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	-
Go_P_St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*
Go_P_St	Bacteroidota	*Bacteroidia*	*Bacteroidales*	-	-
Go_P_St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
Go_P_St	Desulfobacterota	-	-	-	-
P_St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
P_St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Eubacterium_xylanophilum _group*
Rw_St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
Go_St	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	-
St	Cyanobacteria	-	-	-	-
St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Marvinbryantia*
St	Firmicutes	*Clostridia*	*vadinBB60_group*	*uncultured_bacterium*	-
St	Firmicutes	*Clostridia*	*Oscillospirales*	*Butyricicoccaceae*	*UCG-009*

Supplementary Table 1: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
St	Firmicutes	*Clostridia*	*Oscillospirales*	*Eubacterium_ coprostanoligenes _group*	*uncultured_bacterium*
St	Firmicutes	*Clostridia*	*Lachnospirales*	-	-
St	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020 _group*
P	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*uncultured*
P	Firmicutes	*Clostridia*	*Clostridia_vadinBB60 _group*	-	-
Go	Proteobacteria	*Alphaproteobacteria*	*Azospirillales*	*Azospirillaceae*	*Azospirillum*
Go	Proteobacteria	*Alphaproteobacteria*	*Azospirillales*	*Azospirillaceae*	-
Go	Proteobacteria	*Alphaproteobacteria*	*Azospirillales*	-	-

Supplementary Table 2: Taxonomic classification of shared and unique seed endophytic bacterial genera in G0 and G3 *Arabidopsis thaliana* Landsberg *erecta* (L*er*) seeds across four substrates. Taxonomic classification of shared and unique seed endophytic bacteria detected in L*er* accession of G0 and G3 cultivated on four different growth substrates: rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Bacterial taxa are presented at five taxonomic ranks: phylum, class, order, family, and genus, based on 16S rRNA gene sequencing.

Shared/Unique	Phylum	Class	Order	Family	Genus
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	-	-
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*uncultured_bacterium*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*
Core	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*
Core	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
Core	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
Core	Firmicutes	*Bacilli*	*Erysipelotrichales*	-	-
Core	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	-
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	-
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*

Supplementary Table 2: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_NK4A136_group*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured*
Core	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*uncultured*
Core	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
Go_P_St_F	Firmicutes	*Clostridia*	*Oscillospirales*	-	-
Go_Rw_St_F	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	-
Rw_St_F	Firmicutes	*Bacilli*	*Lactobacillales*	-	-
Go_Rw_P_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiracea*	*Roseburia*
Go_Rw_P_F	Desulfobacterota	-	-	-	-
Go_Rw_P_F	Firmicutes	*Clostridia*	*Oscillospirales*	*Butyricicoccaceae*	*UCG-009*
Go_Rw_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
Go_Rw_F	Firmicutes	*Clostridia*	*Clostridia_vadinBB60_ group*	*uncultured_bacterium*	-
Rw_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Eubacterium_xylanophilum_group*
Rw_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020_group*
Rw_F	Firmicutes	*Clostridia*	-	-	-
Rw_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
Rw_F	Firmicutes	*Clostridia*	*Lachnospirales*	-	-
Rw_F	Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Candidatus_Arthromitus*
F	Cyanobacteria	-	-	-	-

Supplementary Table 2: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
F	Firmicutes	-	-	-	-
F	Firmicutes	*Clostridia*	*Clostridia_vadinBB60_ group*	-	-
F	Firmicutes	*Clostridia*	*Oscillospirales*	*Eubacterium_ coprostanoligenes_group*	*uncultured_bacterium*
Go_Rw_P_St	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	-
Go_St	Deferribacterota	*Deferribacteres*	*Deferribacterales*	-	-
Rw	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Marvinbryantia*
Rw	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*uncultured*
Rw	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*Colidextribacter*
Rw	Firmicutes	*Clostridia*	*Clostridia_UCG-014*	*uncultured_bacterium*	-

Supplementary Table 3: Taxonomic classification of shared and unique seed endophytic bacterial genera in G0 and G3 *Arabidopsis thaliana* Bayreuth (Bay) seeds across four substrates. Taxonomic classification of shared and unique seed endophytic bacteria detected in the Bay accession of G0 seeds and G3 seeds cultivated on four different growth substrates of rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Bacterial taxa are presented at five taxonomic ranks: phylum, class, order, family, and genus, based on 16S rRNA gene amplicon sequencing.

Shared/Unique	Phylum	Class	Order	Family	Genus
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*uncultured_bacterium*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*
Core	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*
Core	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
Core	Deferribacterota	-	-	-	-
Core	Deferribacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
Core	Firmicutes	*Bacilli*	*Erysipelotrichales*	-	-
Core	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	-
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*

Supplementary Table 3: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_NK4A136_group*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured*
Core	Firmicutes	*Clostridia*	*Oscillospirales*	-	-
Core	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*uncultured*
Core	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
Go_P_St_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	-
Rw_St_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
Go_St_F	Firmicutes	*Clostridia*	*Oscillospirales*	*Butyricicoccaceae*	*UCG-009*
Go_St_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
St_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Eubacterium_xylanophilum_group*
St_F	Firmicutes	*Clostridia*	*Oscillospirales*	*Eubacterium_ coprostanoligenes_group*	*Uncultured bacterium*
Go_P_F	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	-
Go_P_F	Deferribacterota	*Deferribacteres*	*Deferribacterales*	-	-
F	Firmicutes	*Clostridia*	*Clostridia_vadin_BB60 group*	*uncultured_bacterium*	-
F	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Tannerellaceae*	*Parabacteroides*
F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020_group*
F	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	-
F	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*Colidextribacter*

Supplementary Table 3: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Marvinbryantia*
F	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Rikenellaceae_RC9_gut_group*
Go_St	Firmicutes	*Clostridia*	*Lachnospirales*	-	-
Go_St	Firmicutes	*Bacilli*	*Lactobacillales*	-	-
Go_Rw_P	Bacteroidota	*Bacteroidia*	*Bacteroidales*	-	-
P	Firmicutes	-	-	-	-
Go	Cyanobacteria	-	-	-	-

Supplementary Table 4: Taxonomic classification of shared and unique seed endophytic bacterial genera in G0 and G3 *Arabidopsis thaliana* Shahdara (Sha) seeds across four substrates. Taxonomic classification of shared and unique seed endophytic bacteria detected in the Sha accession of G0 seeds and G3 seeds cultivated on four different growth substrates of rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Bacterial taxa are presented at five taxonomic ranks: phylum, class, order, family, and genus, based on 16S rRNA gene amplicon sequencing.

Shared/Uniqued	Phylum	Class	Order	Family	Genus
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Uncultured bacterium*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
Core	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*
Core	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*
Core	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
Core	Deferribacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
Core	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
Core	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae NK4A136_group*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*

Supplementary Table 4: Continued

Shared/Uniqued	Phylum	Class	Order	Family	Genus
Core	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured*
Core	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*uncultured*
Core	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
Go_Rw_St_F	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	-
Go_Rw_St_F	Firmicutes	*Clostridia*	*Oscillospirales*	-	-
Go_Rw_St_F	Bacteroidota	*Bacteroidia*	*Bacteroidales*	-	-
Go_Rw_P_F	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	-
Go_Rw_P_St	Firmicutes	*Bacilli*	*Erysipelotrichales*	-	-
Go_Rw_P_St	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	-
Go_Rw_P_St	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	-
Go_Rw_P_St	Desulfobacterota	-	-	-	-
Go_Rw_St	Deferribacterota	*Deferribacteres*	*Deferribacterales*	-	-
Rw_P	Firmicutes	*Bacilli*	*Lactobacillales*	-	-
Go_P	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
St	Firmicutes	*Clostridia*	*Oscillospirales*	*Eubacterium_ coprostanoligenes_group*	*uncultured_bacterium*
P	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	-
P	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
P	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020_ group*
Rw	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*uncultured*
Rw	Cyanobacteria	-	-	-	-
Rw	Firmicutes	*Clostridia*	*Lachnospirales*	-	-

Supplementary Table 5: Core seed endophytic bacterial genera shared across all *Arabidopsis thaliana* accessions and substrates at G0 and G3 generations. Endophytic bacterial genera consistently detected in seeds of all four A. thaliana accessions: Columbia-0 (Col-0), Landsberg *erecta* (L*er*), Bayreuth (Bay), and Shahdara (Sha), in both G0 and G3 seeds across four growth substrates of rockwool (Rw), potting substrate (P), gamma-sterilized field soil (St), and non-sterile field soil (F). Taxa were identified by 16S rRNA gene amplicon sequencing and are reported at five taxonomic ranks: phylum, class, order, family, and genus.

No.	Phylum	Class	Order	Family	Genus
Shared all accessions and substrates					
1	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*uncultured_bacterium*
2	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
3	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
4	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
5	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
6	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*
7	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
8	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacteraceae*
9	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
10	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
11	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
12	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
13	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
14	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
15	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*
16	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured*
17	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
18	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_NK4A136_group*
19	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*

Supplementary Table 5: Continued

No.	Phylum	Class	Order	Family	Genus
20	Firmicutes	*Clostridia*	*Lachnospirales*	*Oscillospiraceae*	*uncultured*
Shared between core of L*er*, Bay and Sha					
1	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*
2	Proteobacteria;	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
3	Desulfobacterota;	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
Shared between core of Col-0, L*er* and Bay					
1	Firmicutes	*Bacilli*	*Erysipelotrichales*	-	-
2	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	-
Shared between core of Col-0 and Bay					
1	Firmicutes	*Clostridia*	*Oscillospirales*	-	-
Unique of Bay core					
1	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
2	Desulfobacterota	-	-	-	-
Unique of Col-0 core					
1	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	-
Unique of L*er* core					
1	Bacteroidota	*Bacteroidia*	*Bacteroidales*	-	-

Supplementary Table 6: Results of DESeq2 differential abundance analysis of seed microbiome taxa between G0 and G3 generations across Arabidopsis accessions and growth substrates. The table includes baseMean (average normalized abundance), $\log_2$ fold change (log2FoldChange), standard error (lfcSE), Wald test statistic (stat), p-value, and adjusted p-value (padj). Additional columns indicate significance, accession, substrate, and full taxonomic classification from Kingdom to Genus. The complete dataset is available at: Supplementary Table 6_Chapter 2

Supplementary Table 7: Taxonomic classification of unique seed endophytic bacteria in G3 *Arabidopsis thaliana* Columbia-0 (Col-0) seeds cultivated in gamma-sterilized field soil (St) and non-sterile field soil (F). Bacterial taxa were identified based on 16S rRNA gene amplicon sequencing and Venn diagram analysis to distinguish taxa unique to each substrate. Taxa are reported at five taxonomic ranks: phylum, class, order, family, and genus.

No.	Phylum	Class	Order	Family	Genus
Gamma sterile field soil derived seeds					
1	Bacteroidota	*Bacteroidia*	*Bacteroidales*	-	-
2	Cyanobacteria	-	-	-	-
3	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
4	Desulfobacterota	-	-	-	-
5	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*
6	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
7	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Marvinbryantia*
8	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
9	Firmicutes	*Clostridia*	*Lachnospirales*	-	-
10	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020_group*
11	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
12	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Eubacterium_xylanophilum_group*
13	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	-
14	Firmicutes	*Clostridia*	*Oscillospirales*	*Butyricicoccaceae*	*UCG-009*
15	Firmicutes	*Clostridia*	*Oscillospirales*	*Eubacterium_coprostanoligenes_group*	*uncultured_bacterium*
16	Firmicutes	*Clostridia*	*Clostridia_vadinBB60_group*	*uncultured_bacterium*	-
17	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	-
18	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
Field soil derived seeds					
1	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	-

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Author contributions

S.S. designed and performed the experiments, analysed the germination data, and wrote the manuscript.

W.P. analysed the microbial amplicon sequencing data, generated the microbial community figures, and contributed to writing the manuscript (Materials and Methods).

P.G. and S.G. assisted with microbial DNA extraction.

M.A.S.A., R.K., and L.B. supervised the project, designed the experiments, and edited the manuscript.

Chapter 3

Seed bacterial volatiles stimulate seed germination and seedling growth under abiotic stresses

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ABSTRACT

Microbial volatile organic compounds (VOCs) are emerging as important long or short distance signals in plant-microbe interactions, capable of influencing plant growth and stress responses without direct contact with plant tissues. In this study, we investigated the effects of VOCs emitted by the seed endophytic bacterium *Pantoea agglomerans* E44, isolated from wild cabbage, on seed germination and early seedling development under abiotic stress conditions in multiple *Arabidopsis thaliana* accessions. Exposure to E44 volatiles significantly enhanced germination, seedling biomass, and root system development under salinity and osmotic stress, with responses varying among accessions, indicating strong genotype-dependent effects. To gain mechanistic insight into these phenotypic responses, we performed RNA-seq analysis of imbibed Arabidopsis seeds prior to radicle emergence under salt stress in the presence of E44 volatiles. Salt stress was the main driver of global transcriptional changes, whereas the direct effect of E44 volatiles alone was limited. Instead, E44 substantially reshaped the transcriptional response to salinity through two main patterns. First, a substantial group of salt-responsive genes was no longer differentially regulated in the presence of E44, suggesting a reduced impact of salt stress on gene expression. These genes were enriched in processes related to ribosome biogenesis, rRNA processing, and protein synthesis. Second, a distinct set of genes responsive to salt stress only in the presence of E44 was enriched in detoxification, redox homeostasis, sulfur metabolism, and other adaptive stress-response pathways. Together, these findings suggest that E44 volatiles promote germination and early seedling development under abiotic stress, while transcriptomic analysis of salt-stressed imbibed seeds indicates that this effect may involve preserving ribosome-related processes and reshaping stress-responsive gene expression toward more stress-adaptive pathways. This study provides new insights into how microbial VOCs regulate early plant development and highlights their potential for improving plant resilience under adverse environmental conditions.

Key words: Microbial volatile organic compounds (VOCs), *Pantoea agglomerans*, *Arabidopsis thaliana*, Seed germination, Seedling growth, Abiotic stress, Transcriptomics

INTRODUCTION

Plants are continuously exposed to various abiotic stresses, such as drought and salinity, which collectively restrict crop growth and yield worldwide (Dos Santos et al., 2022). Climate change is further worsening these effects on plants due to more frequent and severe extreme environmental events, increasing the threat to global food security (Chaudhry & Sidhu, 2022). Plant breeding and genetic engineering have been established as key approaches for enhancing plant stress tolerance; however, at present, these strategies alone may be insufficient to address the rapidly changing environmental conditions encountered in agricultural systems (Lawlor, 2012; Xiong et al., 2022). Therefore, genetic strategies should be complemented by additional approaches to enhance crop resilience under increasingly extreme and unpredictable environments (Prado et al., 2025). Among these complementary strategies, plant-associated microorganisms offer a promising opportunity, representing a significant yet underexplored source of functional diversity that can support plant growth and improve tolerance to abiotic stress (Taglialegna, 2025; Tripathi et al., 2025). Microbiome-based strategies can be integrated with breeding and genetic engineering to enhance crop performance under complex environmental conditions while reducing dependence on chemical inputs, contributing to the development of sustainable and resilient agricultural systems (Portal-Gonzalez et al., 2025; Shen et al., 2024; Zhao et al., 2025).

Endophytic bacteria, microbes that colonize internal plant tissues without causing disease, are key members of the plant microbiome that promote growth and stress resilience (Ercole et al., 2025; Liu et al., 2017). These microorganisms enhance plant growth and stress adaptation by improving nutrient and water uptake, strengthening antioxidant defences, and activating stress-responsive signalling pathways (Nikalje et al., 2026). Their beneficial effects are mediated through the production of bioactive metabolites, including volatile organic compounds (VOCs), phytohormones, antibiotics, and enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, as well as through modulation of endogenous hormone pathways involving abscisic acid, auxin, and ethylene (Compant et al., 2019; Das et al., 2025; Ek-Ramos et al., 2019; Godara & Ramakrishna, 2022; Sherzad et al., 2025). Beyond these immediate physiological effects, endophytes may also induce epigenetic modifications that create molecular memory states, enabling faster and stronger responses to later stresses (Devesa-Aranguren et al., 2025; Lata et al., 2018). These effects are especially important during early developmental stages, such as seed germination and seedling establishment, when plants are highly sensitive to environmental stress (Finch-Savage & Bassel, 2016).

Within the broader endophytic community, seed endophytes represent a uniquely important yet underexplored group (Nelson, 2018; Romão et al., 2025; Wang & Zhang, 2023). These microorganisms are vertically transmitted across generations, establishing the earliest plant-microbe interactions during seed development and potentially transferring beneficial traits from parent plants to their offspring (Khan et al., 2025; Shade et al., 2017). As a result, seed endophytes are well positioned to influence early developmental processes (Pal et al., 2022; Walitang et al., 2017). Among the mechanisms employed by seed endophytes, VOCs act as important long-distance signals that mediate interactions between plants and associated microorganisms (Farag et al., 2013; Marzouk et al., 2021). These compounds influence plant physiological and molecular processes, including stress-related gene expression, hormone signalling, and metabolic regulation associated with early growth, such as water uptake, cell expansion, and energy metabolism (Fincheira et al., 2017; Sziderics et al., 2007; Tahir et al., 2017b). Through these effects, VOCs can contribute to key developmental processes such as germination, early root development, and seedling establishment, particularly under stressful conditions (Türksoy et al., 2025; Tyc et al., 2020).

Previous studies have shown that microbial VOCs can reprogram plant gene expression and activate pathways linked to stress adaptation and growth (Gao et al., 2022). Transcriptomic analyses have shown that transcription factor families such as Heat Shock Factors (HSFs), NAC (No Apical Meristem, Arabidopsis Transcription Activation Factor, and Cup-Shaped Cotyledon) transcription factors, and WRKY transcription factors are enriched in VOC-exposed plants, suggesting key roles in mediating VOC responses (Chang et al., 2024). Microbial VOCs have also been shown to improve abiotic stress tolerance by regulating genes related to hormone signalling, redox balance, defence responses, and energy metabolism, leading to reduced ROS accumulation and cellular damage under salinity (Nguyen et al., 2025a). In addition, bacterial VOCs can upregulate genes associated with plant growth and development (He et al., 2024), as well as pathways related to translation, biosynthesis, and hormone regulation that may support early growth processes (Almeida et al., 2022). Collectively, these findings indicate that microbial VOCs influence regulatory and metabolic networks that coordinate plant defence and growth under stress conditions.

Despite growing interest in microbial VOCs, studies specifically examining VOCs released by seed endophytes under abiotic stress remain limited. One of the few seed-specific studies, Tyc et al. (2020), showed that VOCs produced by the seed endophyte *Pantoea agglomerant* E44, isolated from wild cabbage seeds, significantly enhanced germination and primary root growth under optimal conditions. This effect was partly attributed to compounds such as dimethyl

disulfide (DMDS), which stimulate auxin-related pathways. These findings highlight the potential of *Pantoea agglomerans* as a beneficial seed-associated bacterium capable of influencing early plant development through VOC-mediated mechanisms. *Pantoea agglomerans* is a widely distributed seed-associated bacterium with known plant growth-promoting potential (Luziatelli et al., 2020; Sanz-Puente et al., 2025). Most studies have focused on its effects following direct inoculation, particularly on pathogen suppression (Poppe et al., 2003; Singh et al., 2020; Xie et al., 2017), whereas the role of its VOCs in mediating plant responses to abiotic stress remains largely unexplored. Microbial VOCs from endophytes in other plant tissues, such as roots and leaves, have been shown to promote plant growth and enhance stress tolerance (Cordovez et al., 2018; Hossain et al., 2019; Jayakumar et al., 2021; Meldau et al., 2013; Narayanasamy & Uthandi, 2024). However, the specific roles of seed endophyte-derived VOCs during germination and early seedling development under abiotic stress remain poorly understood. Whether these VOC-mediated effects are conserved across plant genotypes and via which molecular mechanisms seed perception of microbial VOCs occurs remain important knowledge gaps. In addition, transcriptomic studies focusing specifically on early developmental stages, particularly germinating or imbibed seeds exposed to microbial VOCs, remain limited. Understanding molecular responses at this developmental stage is important because stress perception during seed imbibition can strongly influence subsequent germination and seedling establishment (Ali & Elozeiri, 2017).

In this study, we used *Arabidopsis thaliana* as a model system to investigate how seeds and seedlings respond to VOCs produced by bacterial seed endophyte *Pantoea agglomerans* E44. We examined the effects of bacterial VOCs on seed germination and early seedling growth under abiotic stress conditions, including drought and salinity. To gain mechanistic insight, we analysed transcriptional responses in imbibed seeds exposed to bacterial volatiles using RNA sequencing, aiming to identify genes and regulatory pathways activated prior to germination. By integrating physiological and transcriptomic analyses, this work provides a framework for understanding how seed-associated bacterial VOCs contribute to plant stress resilience during germination and early development. Ultimately, these findings may support innovative applications such as the development of seed coatings based on microbes or VOC-based biostimulants to enhance crop performance under challenging environmental conditions.

MATERIALS AND METHODS

Plant materials

Seeds from four different *Arabidopsis thaliana* accessions were used: Columbia-0 (Col-0), Shahdara (Sha), Landsberg *erecta* (L*er*), and Bayreuth (Bay). Plants were cultivated on rockwool in a randomized design in a climate-controlled growth chamber at Unifarm, Wageningen University & Research, maintained at 20°C during the day and 18°C at night, with a 16-hour light/8-hour dark photoperiod. Seeds were harvested in January 2022. For each accession, seeds from four individual plants grown under the same conditions were pooled to generate a single seed batch, which was used throughout all experiments. Multiple technical replicates were established using seeds from this pooled batch. After harvesting, seeds were stored in a controlled cabinet at 20 °C and 30% relative humidity until further use.

Bacterial strains and preparation

The seed endophytic bacterial *Pantoea agglomerans* E44, originally isolated from wild cabbage seeds as described in Tyc et al. (2020), was used. The strain was kindly provided by Dr. Paolina Garbeva (NIOO-KNAW). In addition, *Escherichia coli* (E. coli) DH5α, a commonly used laboratory strain, was included as a non-endophytic bacterial strain for comparison. Bacterial strains were recovered from glycerol stocks, streaked onto $1/10^{th}$ tryptic soy broth agar (TSBA) (5.0 g/L NaCl; 1.0 g/L KH_2PO_4; 3 g/L Oxoid Tryptic Soy Broth; 20 g/L BACTO agar; and 1L demineralized water, pH 6.5). Plates were incubated at 28°C for 48 hours. Single colonies were inoculated into 3 mL of liquid $1/10^{th}$ tryptic soy broth (TSB) (same composition as above, without agar; pH 6.5) and incubated overnight at 28°C with shaking at 190 rpm. Cells from the overnight culture were harvested by centrifugation at 4,000 rpm for 5 min, and the resulting pellets were washed twice and resuspended in 10 mM phosphate buffer (pH 6.5). Final bacterial solutions were adjusted to an optical density of OD_{600} = 0.005 in 10 mM phosphate buffer (pH 6.5). Sterile phosphate buffer was used as a control, representing the absence of bacterial volatiles.

Seed germination assay in response to bacterial volatiles

To evaluate the effects of volatiles emitted by seed endophytic bacteria on *Arabidopsis thaliana* seed germination, a germination assay was conducted under controlled laboratory conditions. Germination was assessed under non-stress conditions using demineralized water, osmotic stress induced by 300 mM D-mannitol, and salinity stress induced by 100 mM NaCl, in the presence or absence of bacterial volatiles.

Bacterial plates preparation

Prior to sowing seeds, bacterial inoculation plates were prepared. Fifty microliters of each bacterial solution as described in "Bacterial strains and preparation", or buffer control were plated onto the lids of 60 × 15 mm round Petri dishes (Greiner Bio-One B.V., NL) containing 10 mL of 1/10th TSBA (pH 6.5). The plates were placed into a square Petri dish (120 × 120 × 70 mm; four round plates fit within one square dish), sealed in a plastic bag, and incubated at 20 °C for 48 hours to allow bacterial growth. At this stage, the bacterial plates were not yet exposed to seeds and were subsequently transferred under sterile conditions to the germination plates in the next assay step.

Dry seed sterilization

Prior to sowing, seeds were surface-sterilized using chlorine gas. In the fume hood, opened Eppendorf tubes containing seeds were placed inside a closed desiccator with a glass beaker containing 100 mL of bleach (sodium hypochlorite) and 3 mL of 37% hydrochloric acid to generate chlorine gas. Seeds were exposed to the gas for 5 hours. After sterilization, the seeds were stored in Eppendorf tubes and in a controlled cabinet at 20°C and 30% relative humidity until further use.

Germination assay

Sterilized seeds were sown onto two layers of blue germination paper placed in 120 x 120 x 17 mm square plates (Greiner Bio-One B.V., NL), pre-soaked with 15 mL of either demineralized water (non-stress condition), 300 mM D-mannitol (osmotic stress), or 100 mM NaCl (salinity stress). One bacterial or control plate was placed in the top-left corner, hereafter referred to as the single-plate setup. Each square plate contained three replicates of approximately 50 seeds of the same accession. Three plates were used per treatment, resulting in a total of nine technical replicates per treatment. Because each plate contained seeds from a single accession, randomization was applied to the position of the plates within the growth chamber. Plates were stacked, and their positions within the stack were randomized daily to minimize positional effects. The experimental setup is shown in Supplementary Fig. 1a. In this setup, bacterial strains were grown separately, and seeds were exposed exclusively to their volatiles without direct bacterial-seed contact. All plates were sealed with parafilm to prevent volatile loss, randomized, and incubated at 22°C under continuous light for 7 days. Images were captured twice daily, in the morning and afternoon, and analysed using the GERMINATOR software package (Joosen et al., 2010). Germination metrics, including the percentage of maximum germination (Gmax) and the time to reach 50% of maximum germination (T50), were calculated. Statistical analysis of

seed germination parameters was performed using one-way ANOVA followed by Fisher's least significant difference (LSD) test at $p < 0.05$.

Seedling growth assay in response to bacterial volatiles

To evaluate the effects of volatiles emitted by seed endophytic bacteria on *Arabidopsis thaliana* seedling growth and root formation, a seedling growth assay was conducted under controlled laboratory conditions. Seedling growth was assessed under non-stress conditions, osmotic stress induced by 150 mM sorbitol, and salinity stress induced by 75 mM NaCl, in the presence or absence of bacterial volatiles.

Bacterial plate preparation

Prior to the seedling growth assay, square plates containing ½ MS medium and an embedded small bacterial Petri dish were prepared. All procedures were carried out in a laminar flow cabinet to maintain sterility. A small round Petri dish (35 × 10 mm; Greiner Bio-One) containing 7 mL of 1/10th TSBA (pH 6.5) was first positioned at the bottom center edge of a 120 × 120 × 17 mm square plate (Greiner Bio-One). Subsequently, 42 mL of molten ½ MS medium (1 g/L MES monohydrate, 10 g/L Daishin agar, 2.2 g/L Murashige and Skoog basal medium, and 1 L demineralized water, pH 5.8) was poured into the square plate, surrounding the small round Petri dish and allowed to solidify in place. The ½ MS medium served either as a control or was supplemented with 75 mM NaCl (salinity stress) or 150 mM sorbitol (osmotic stress). After solidification, 20 µL of *Pantoea agglomerans* E44 or *Escherichia coli* DH5α suspension (OD_{600} = 0.005), or 10 mM phosphate buffer (pH 6.5), was inoculated onto the TSBA in the small round Petri dishes. *Escherichia coli* DH5α, a non-endophytic bacterial strain, and 10 mM P-buffer, which served as a non-bacterial volatile control, were included for comparison. Preparation of the bacterial suspensions is described in the section "Bacterial strains and preparation." The assembled plates were incubated at 20°C for 48 hours to allow bacterial growth prior to the seedling growth assay.

Wet seed sterilization and pre-growth seedling

Seeds were subjected to wet sterilization to remove the epiphyte from the seed surface. Seeds were placed in a 2 mL Eppendorf tube and incubated with 1 mL of 0.1% (v/v) sodium hypochlorite mixed with 0.1% (v/v) Tween 80. The tube was gently tapped to mix, ensuring minimal foaming, followed by a 1 minute incubation. After centrifugation the supernatant was removed using sterile tips. The seeds were then rinsed five times with 1 mL of sterile demineralized water per wash. This sterilization protocol was optimized in preliminary pilot experiments in which multiple sterilization conditions were compared to ensure effective removal of epiphytic microbes while maintaining seed-associated endophytes.

Sterilized seeds were sown on square plates of 120x120x17mm (Greiner Bio-one) containing 42 mL of ½ MS medium (pH 5.8). All plates were sealed with Parafilm and stratified at 4 °C in darkness for 48 hours. After stratification, plates containing seeds were placed vertically on racks at a 70° angle in a growth chamber set to 22°C under light conditions. Seeds were allowed to germinate for 3 day to obtain the pre-growth seedling. Under sterile conditions, these seedlings were then transferred onto the previously prepared square ½ MS plates containing the small bacterial Petri dish for the subsequent seedling growth assay.

Seedling growth assay

Five three-day-old seedlings, each with a root length of approximately 0.5 cm, were transferred onto the top portion of square ½ MS plates containing the small bacterial Petri dish that had been incubated for 48 hours as previously described. The same procedure was applied to ½ MS plates supplemented with 75 mM NaCl (salinity stress) or 150 mM sorbitol (osmotic stress). The experimental setup is shown in Supplementary Fig. 1b. Five replicate plates were used per treatment. All plates were sealed with Parafilm to prevent volatile loss and were placed vertically on racks at a 70° angle in a growth chamber set to 22°C under light conditions for 13 days. Pictures of the seedlings were taken on the first and tenth days of the assay. Root images were analysed using RootNav 1.8.1 (Pound et al., 2013) to determine primary root length, lateral root length, and lateral root count per seedling. Shoot and root fresh weights were measured per plate by pooling five seedlings due to the small size of individual seedlings. Statistical analysis of seedling growth parameters was performed using one-way ANOVA followed by Fisher's least significant difference (LSD) test at $p < 0.05$.

RNA Sequencing experiment

Imbibed *Arabidopsis thaliana* (L*er* accession) seeds exposed to bacterial volatiles produced by seed endophytic bacterial *Pantoea agglomerans* E44 prior to radicle emergence were sampled for RNA-seq analysis to investigate transcriptional responses to bacterial volatile exposure.

Germination assay and RNA-seq sampling

Preliminary germination assays were performed to determine appropriate sampling time points for RNA-seq analysis. L*er* seeds were surface-sterilized using chlorine gas as described previously. Sterilized seeds were sown onto two layers of blue germination paper placed in 120 x 120 x 17 mm square plates (Greiner Bio-One B.V., The Netherlands). The germination papers were pre-soaked with 10 mL either demineralized water (non-stress condition) or 100 mM NaCl (salinity stress condition). The 100 mM NaCl concentration was chosen to

match the salinity treatment used in a previous germination assay investigating the effects of bacterial volatiles on *A. thaliana* seed germination. A double-plate set-up was used to increase exposure of seeds to bacterial volatiles without direct contact. Two bacterial or control plates were positioned in the top-left and bottom-right corners of each germination plate. Bacterial plates containing *Pantoea agglomerans* E44 in round Petri dishes were prepared as described in the "Seed germination assay in response to bacterial volatiles." Each square plate contained two technical replicates of approximately 50 seeds each, and four plates were used per treatment, resulting in eight technical replicates per treatment. The experimental setup is shown in Supplementary Fig. 1c. All plates were sealed with parafilm, randomized, and incubated at 22°C under continuous light for 7 days. Germination was monitored to determine the time of first radicle emergence under both non-stress and salinity stress conditions. First germination occurred at approximately 36 hours after imbibition in demineralized water and 56 hours after imbibition in 100 mM NaCl. Based on these observations, seeds were sampled for RNA-seq at 30 hours (non-stress) and 48 hours (salinity stress) after imbibition, prior to radicle protrusion. A consistently higher germination rate in volatile-exposed seeds compared to controls was confirmed under the double-plate set-up (Supplementary Fig. 2). For RNA-seq sampling, the germination assay was repeated under both demineralized water and 100 mM NaCl conditions, and imbibed seeds were harvested at the selected time points. Three technical replicates per treatment were collected, immediately frozen in liquid nitrogen, and stored at -80 °C until RNA extraction.

RNA extraction from imbibed seeds

Seeds prior to germination, imbibed in demineralized water or 100 mM NaCl, were used for RNA extraction. Total RNA was isolated using a modified hot borate protocol as described by Maia et al. (2011). For each treatment, three technical replicates were prepared, each consisting of approximately 200 imbibed seeds (≈5 mg). Seeds were ground to a fine powder and mixed with RNA extraction buffer preheated to 80°C, followed by incubation with proteinase K for 15 min at 42°C. RNA was then precipitated with lithium chloride (LiCl) at 4°C overnight. Samples were centrifuged, the supernatant was discarded, and the RNA pellets were washed with 70% ethanol and resuspended in 20 µL of RNase-free water. RNA concentration and purity were measured using a NanoDrop spectrophotometer. RNA integrity and quality control were further assessed by the sequencing provider. Library preparation and transcriptome sequencing were conducted by Novogene (UK) on a Novaseq 6000 platform.

RNA-Seq data analysis pipeline

RNA sequencing data were obtained in FASTQ format. Initial quality assessment of raw sequencing reads was performed using FastQC (Andrews, 2010), and high-quality reads were aligned to the *Arabidopsis thaliana* reference genome TAIR10.1 (GCF_000001735.4) using HISAT2 (Kim et al., 2019). Aligned reads were assembled and quantified at the gene level using StringTie (Pertea et al., 2015), generating raw read count matrices for downstream analyses.

Differential gene expression analysis was conducted in R using the DESeq2 package (Love et al., 2014). Raw count matrices were filtered to remove genes with zero counts across all samples, and genes with fewer than 10 total counts were excluded prior to normalisation. Pairwise comparisons were performed across four experimental contrasts: buffer_Demi vs E44_Demi, buffer_NaCl vs E44_NaCl, buffer_Demi vs buffer_NaCl, and E44_Demi vs E44_NaCl. Read counts were normalised using the median-of-ratios method implemented in DESeq2, and variance-stabilising transformation (VST) was applied for visualisation and sample-level analyses. Genes with an adjusted p-value (false discovery rate, FDR) < 0.05 and an absolute log2 fold change (|log2FC|) > 0.58 (equivalent to a 1.5-fold change) were defined as significantly differentially expressed genes (DEGs), unless otherwise stated. Gene annotation was performed by integrating DESeq2 results with gene feature information extracted from the TAIR10 GFF3 annotation file, including gene identifiers, gene names, genomic coordinates, and functional descriptions.

To assess overall sample quality and transcriptional variation, principal component analysis (PCA) was performed on VST-normalised counts using the plot PCA function in DESeq2, with sample labels assigned based on treatment and stress condition metadata. In addition, sample-to-sample Euclidean distances were calculated from VST-normalised counts and visualised as a hierarchical clustering heatmap using the ComplexHeatmap package (Gu et al., 2016). Differential expression patterns for each contrast were further visualised using volcano plots generated with ggplot2 (Wickham, 2016), in which genes were classified into multiple significance categories based on combinations of adjusted p-value thresholds (adjusted p-value < 0.01, 0.05, and 0.10) and fold-change cut-offs (|log2FC| > 0, 0.58, 1, and 2). Furthermore, overlap of significant DEGs among the four contrasts was examined using Venn diagram analysis implemented with the VennDetail package, through which unique and shared gene sets were identified, visualised, and exported for downstream interpretation.

To address three specific biological questions, DEG sets were then combined across contrasts to define biologically meaningful gene sets. First, the E44-

responsive gene set (E44RGs) was defined as the intersection of DEGs from buffer_Demi vs. E44_Demi and buffer_NaCl vs. E44_NaCl, thereby capturing genes that respond to E44 volatile exposure independently of salinity. Second, the salt-responsive gene set (SRGs) was defined as the intersection of DEGs from buffer_Demi vs. buffer_NaCl and E44_Demi vs. E44_NaCl, representing genes that respond to salt stress independently of E44 treatment. Third, a rescue analysis was performed to identify genes whose salt-induced transcriptional changes were attenuated by E44 volatile exposure. Rescued genes were therefore defined as DEGs significant in the buffer_Demi vs buffer_NaCl comparison, but not significant in the E44_Demi vs E44_NaCl comparison, using the same thresholds of adjusted p-value < 0.05 and |log2FC| > 0.58.

Functional enrichment analysis was performed using the clusterProfiler package (Yu et al., 2012) to identify overrepresented Gene Ontology (GO) terms among DEG sets. GO terms associated with biological processes (BP), molecular functions (MF), and cellular components (CC) were identified using the org.At.tair.db annotation database. All enrichment analyses applied the Benjamini–Hochberg correction for multiple testing, and enriched terms were considered significant at adjusted p-value < 0.05 and q-value < 0.05.

RESULTS

The effects of seed bacterial volatiles on Arabidopsis seed germination under abiotic stress are genotype dependent

To study the effect of volatiles from bacterial seed endophyte *Pantoea agglomerans* E44 (hereafter E44) on seed germination, we measured maximum germination percentage (Gmax) and time to 50% germination (T50) of *Arabidopsis thaliana* seeds exposed to E44 volatiles, P-buffer (control; non-bacterial volatile), or volatiles from Escherichia coli DH5α, a non-endophytic bacterial strain, under non-stress or abiotic stress conditions (salinity and osmotic stress). Under non-stress conditions (demineralized water), E44 volatiles had little effect on Gmax, which was generally high across accessions, with Col-0, L*er*, and Sha reaching approximately 98%, whereas Bay seeds germinated up to around 80% regardless of treatment (Fig. 1a). In contrast, Col-0 and Sha seeds exposed to *E. coli* volatiles exhibited a shorter T50 than seeds exposed to the P-buffer control, indicating faster germination, whereas E44 volatiles had no effect on T50 relative to the P-buffer control (Supplementary Fig. 3a).

Under salinity stress (100 mM NaCl), E44 volatiles significantly enhanced germination in most accessions (Fig. 1b). In L*er*, exposure to E44 volatiles

increased Gmax to 88%, compared with 74% in the P-buffer control and 63% for *E. coli*. In Bay, seeds exposed to E44 showed a significant increase in germination (33%) relative to the control (18%). In Sha, exposure to both E44 and *E. coli* volatiles significantly increased Gmax relative to the P-buffer control, reaching 88% and 81%, respectively, compared with 65% in the P-buffer treatment. No significant difference was observed between the two bacterial volatile treatments. Col-0 seeds were largely unaffected by any of the volatile treatments. Notably, seeds of L*er* and Bay exposed to E44 also germinated faster than both *E. coli* and P-buffer control, showing a corresponding increase in both germination percentage and rate (Supplementary Fig. 3b). These results highlight the ability of E44 volatiles to enhance seed germination under salinity stress in an accession-dependent manner.

Under osmotic stress (300 mM mannitol), the effect of bacterial volatiles on seed germination was also accession-dependent (Fig. 1c). Most accessions exhibited very low germination (<40%) under the osmotic stress imposed independent of the volatile treatment, except for L*er*, which maintained high germination (>80%). Exposure to E44 volatiles significantly increased Gmax only in the Sha accession, whereas Col-0 seeds showed reduced germination when exposed to E44. L*er* and Bay seeds did not show significant changes in germination among treatments. T50 could only be determined for L*er* due to its higher germination percentage (above 50%), but no significant differences among volatile treatments were observed (Supplementary Fig. 3c). These results indicate that E44 volatiles can enhance seed germination under osmotic stress only in the Sha accession. Overall, these findings suggest that the effect of E44 volatiles on seed germination under abiotic stress depends on both the type of stress and the plant genotype.

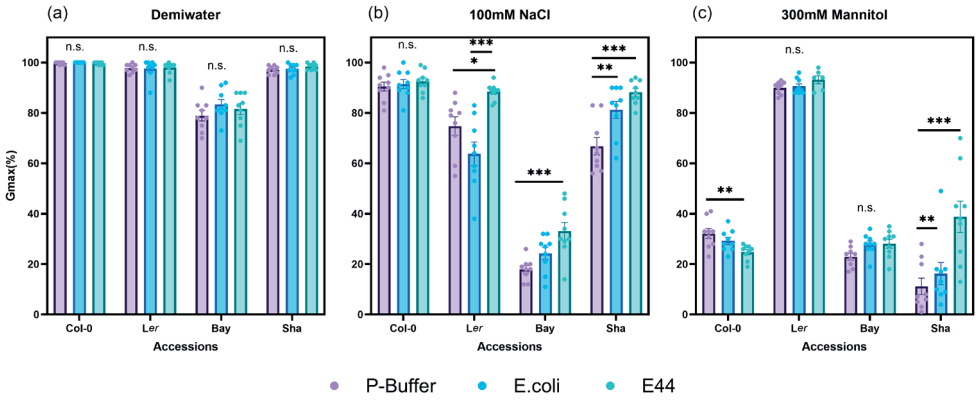


Figure 1: E44 bacterial volatiles enhance seed germination under abiotic stress in a genotype-dependent manner. Maximum germination percentage (Gmax) of seeds from four *Arabidopsis thaliana* accessions (Col-0, L*er*, Bay, and Sha) in the presence or absence of bacterial volatiles under non-stress and abiotic stress conditions. Seeds were exposed to three treatments: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* (non-endophytic bacterial strain), and volatiles produced by *Pantoea agglomerans* E44, a bacterial strain isolated from wild cabbage seeds. Germination was assessed under three conditions: (a) non-stress conditions with demineralized water, (b) salinity stress with 100 mM NaCl, and (c) osmotic stress with 300 mM mannitol. Data are shown as mean ± standard error of the mean (SEM). Statistical differences among volatile treatments within each accession were analysed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); n.s., not significant.

Seed bacterial endophytes enhance seedling growth under abiotic stress in a genotype-dependent manner

To investigate the effects of volatiles from the bacterial seed endophyte *Pantoea agglomerans* E44 on *Arabidopsis thaliana* seedling growth, we measured root and shoot biomass (fresh weight) after exposure to E44 volatiles, P-buffer (non-bacterial volatile; control), or volatiles from *Escherichia coli* DH5α, a non-endophytic bacterial strain, under non-stress (½ MS medium) and abiotic stress conditions (salinity and osmotic stress). Representative images of seedlings from the different accessions (Col-0, L*er*, Bay, Sha) are shown in the Supplementary Figs. 4-7. Under non-stress conditions (½ MS medium), seedling root and shoot fresh weights did not differ significantly among the different treatments. However, responses varied among accessions, with Sha seedlings consistently exhibiting the highest biomass accumulation, showing greater root and shoot fresh weight than the other accessions (Supplementary Fig. 8). Overall, these results indicate that under non-stress conditions, bacterial volatiles have minimal effect on seedling biomass, and differences are primarily determined by the genetic background of the accession.

Under salinity stress (½ MS medium supplemented with 75 mM NaCl), root and shoot seedling fresh weight were reduced compared with non-stress controls in ½ MS medium, regardless of the presence or absence of bacterial volatiles (Fig. 2a-b; Supplementary Fig. 8). Overall, exposure to bacterial volatiles increased both root and shoot fresh weight relative to the non-bacterial control (P-buffer). In Col-0, L*er*, and Bay, seedlings exposed to either E44 or *E. coli* volatiles showed significantly higher biomass than those in the P-buffer control, but no significant difference was observed between E44 and *E. coli*. In Sha, only seedlings exposed to E44 exhibited a significant increase in root and shoot fresh weight relative to P-buffer control (Fig. 2a-b). These results indicate that E44 volatiles enhance seedling growth under salinity stress, with the effect varying among accessions.

Similarly, under osmotic stress (½ MS medium supplemented with 150 mM sorbitol), root and shoot seedling fresh weight were reduced compared with non-stress controls (Fig. 2c-d; Supplementary Fig. 8). Exposure to E44 volatiles significantly increased biomass in both root and shoot in Bay and Sha seedlings. In contrast, *E. coli* volatiles increased root biomass in Col-0 compared with the P-buffer control, while no significant effects were observed for E44 in Col-0 or L*er* accessions (Fig. 2c). These findings indicate that E44 volatiles can enhance seedling growth under osmotic stress, with the response being accession-dependent. Together, these results indicate that volatiles from the bacterial endophyte E44 can enhance seedlings root and shoot biomass under both salinity and osmotic stress. However, the effect varies among accessions, highlighting the potential of E44 to mitigate growth reduction caused by abiotic stress in a genotype-dependent manner.

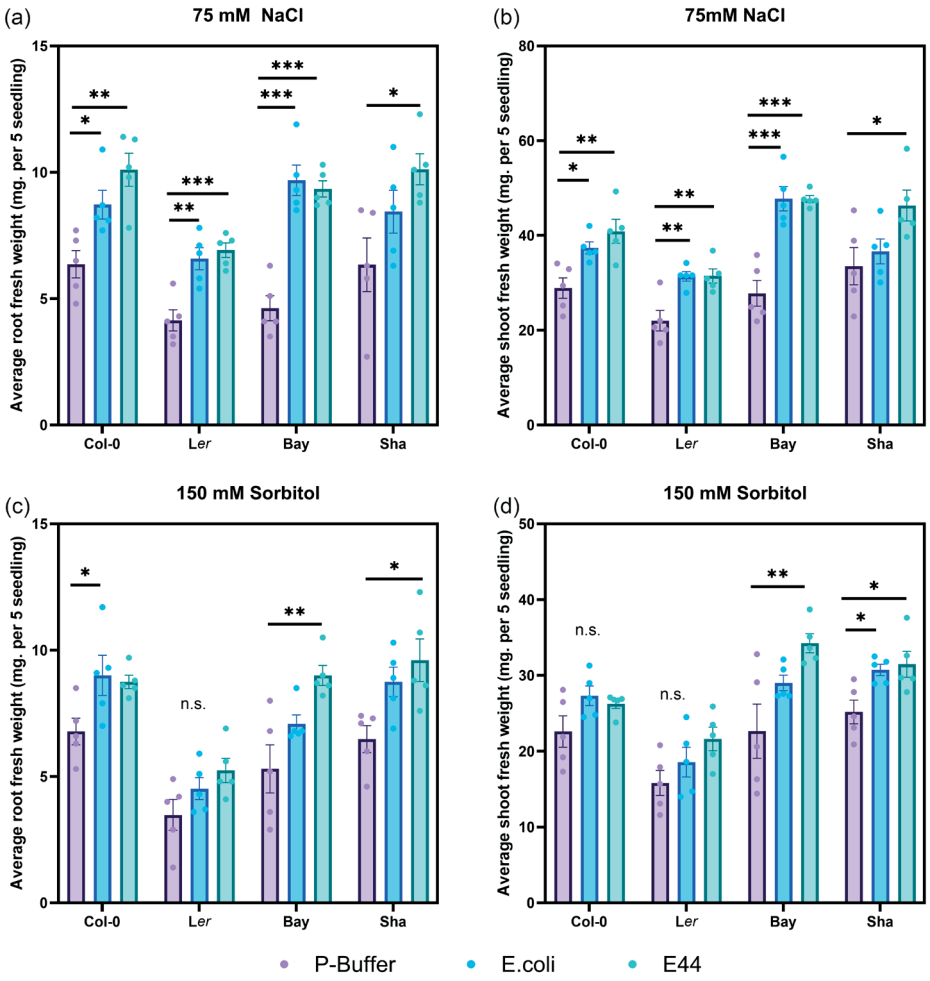


Figure 2: E44 bacterial volatiles enhance seedling root and shoot growth under abiotic stress in a genotype-dependent manner. Three-day-old seedlings from four *Arabidopsis thaliana* accessions (Col-0, L*er*, Bay, and Sha) were transferred to ½ MS medium with or without stress and grown vertically for 13 days. Seedlings were exposed to three treatments: P-buffer (non-bacterial volatile control), volatiles produced by *Escherichia coli* DH5α (a non-endophytic bacterial strain), and volatiles produced by *Pantoea agglomerans* E44, a bacterial strain isolated from wild cabbage seeds. Seedling root (a, c) and shoot (b, d) fresh weight were measured under salinity stress (½ MS medium supplemented with 75 mM NaCl; a-b) and osmotic stress (½ MS medium supplemented with 150 mM sorbitol; c-d). Data are presented as mean ± standard error of the mean (SEM). Statistical differences among volatile treatments within each accession were analysed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); n.s., not significant.

E44 bacterial volatiles enhance seedling root system development under abiotic stress

Based on the observed positive effects of E44 volatiles on seedling biomass, we further investigated their role in root system development. *Arabidopsis thaliana* seedlings were exposed to E44 volatiles, P-buffer (non-bacterial volatile; control), or volatiles from *Escherichia coli* DH5α, a non-endophytic bacterial strain, under non-stress (½ MS medium) and abiotic stress conditions (salinity and osmotic stress). Representative images of seedlings from the different accessions (Col-0, L*er*, Bay, Sha) are shown in the Supplementary Figs. 4-7.

Under non-stress conditions (½ MS medium), primary root length was similar across treatments in all accessions (Fig. 3a). In contrast, lateral root number and total root length were influenced by bacterial volatiles in a genotype-dependent manner. In L*er*, E44 volatiles significantly increased lateral root number and total root length compared with the P-buffer control, whereas *E. coli* volatiles also enhanced these traits, but the effect was smaller than that of E44 (Fig. 3b-c). In Sha, total root length was greater in seedlings exposed to E44 volatiles compared with the P-buffer control (Fig. 3b), indicating that E44 can promote overall root growth under non-stress conditions.

Under salinity stress (½ MS supplemented with 75 mM NaCl), specific root traits were also affected in an accession-specific manner. Primary root length was largely unaffected by E44 volatiles across accessions, while *E. coli* volatiles increased primary root length in L*er*, Bay, and Sha relative to the P-buffer control or E44 (Fig. 3d). Lateral root number was significantly enhanced by both E44 and *E. coli* volatiles in Col-0 and Bay, but in L*er* and Sha only *E. coli* volatiles increased lateral root number (Fig. 3e). Average total root length, which integrates the length primary and lateral roots, was consistently increased by both bacterial volatiles across all accessions, with E44 showing higher effect in all accession, highlighting its ability to promote overall root growth under salt stress conditions (Fig. 3F).

Under osmotic stress (½ MS supplemented with 150 mM sorbitol), E44 volatiles significantly increased primary root length in Bay and Sha (Fig. 3g), and increased lateral root number in Bay, Sha, and L*er* (Fig. 3h). In Col-0, only *E. coli* volatiles promoted lateral root number and primary root length (Fig. 3g-h). Total root length was generally increased by E44 volatiles in all accessions except Col-0, where *E. coli* volatiles resulted in greater total root length than E44 or the P-Buffer control (Fig. 3i). Overall, these results indicate that E44 volatiles consistently enhance total root length, supporting a robust effect on overall root system development under both salinity and osmotic stress. While effects on primary

root length and lateral root number were more variable and accession-dependent, E44 volatiles reliably improved overall root growth in most accessions. Although *E. coli* volatiles also promoted root growth in some accessions, the candidate endophyte E44 displayed overall higher and more consistent effects, highlighting its potential as a beneficial seed endophyte for enhancing root architecture under abiotic stress.

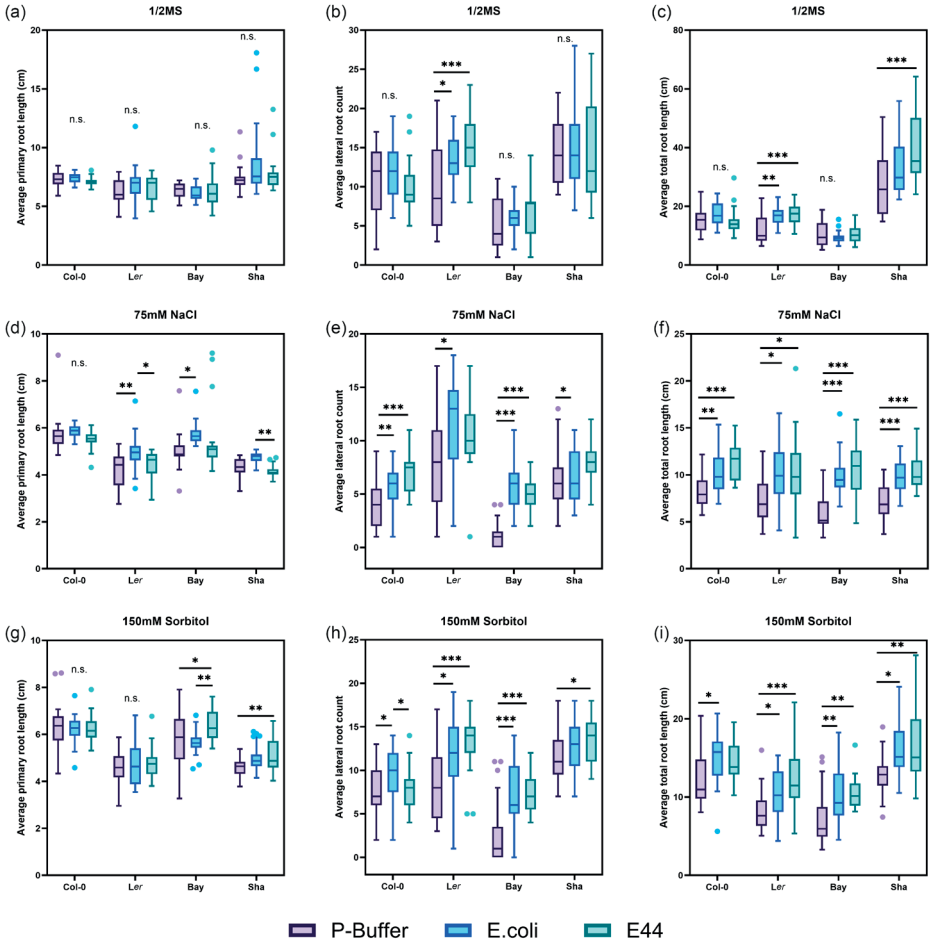


Figure 3: E44 bacterial volatiles enhance root system development under abiotic stress in a genotype-dependent manner. Three-day-old seedlings from four *Arabidopsis thaliana* accessions (Col-0, L*er*, Bay, and Sha) were transferred to ½ MS medium with or without stress and grown vertically for 13 days. Seedlings were exposed to three treatments: P-buffer (non-bacterial volatile control), volatiles produced by *Escherichia coli* DH5α (a non-endophytic bacterial strain), and volatiles produced by *Pantoea agglomerans* E44, a bacterial strain isolated from wild cabbage seeds. Average primary root length (a, d, g), lateral root number (b, e, h), and total root length (c, f, i) were measured under non-stress conditions (½ MS; a-c), salinity stress (½ MS supplemented with 75 mM NaCl; d-f), and osmotic stress (½ MS supplemented with 150 mM sorbitol; g-i). Data are presented as box plots showing the median (center line), interquartile range (box, 25th-75th percentiles), and whiskers representing the minimum and maximum values. Statistical differences among treatments within each accession were analysed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***); n.s., not significant.

Exposure to E44 volatiles led to minor transcriptional changes in salt-stress germinating seeds

As E44 showed accession-specific effects on seed germination under salinity stress, we investigated whether these effects reflect changes at the transcriptional level. RNA-seq was performed on imbibed seeds of the *Arabidopsis thaliana* L*er* accession, selected based on its pronounced responsiveness to E44 volatiles. Specifically, this accession showed higher germination under salinity following exposure to E44 volatiles compared to the P-buffer control (non-bacterial volatile) and volatiles from *Escherichia coli* DH5α (*E. coli*) (Fig. 1b), and also exhibited increased germination under salinity upon E44 volatile exposure in the double-plate setup (Supplementary Fig. 2). Seeds were sampled prior to radicle emergence to capture early transcriptional changes associated with germination. Specifically, seeds were collected after 30 hours of imbibition in demineralized water (non-stress) and 48 hours in 100 mM NaCl (salt stress), with or without E44 volatile exposure, and used for RNA extraction and sequencing (for details on seed sampling timepoints see material and methods).

Principal component analysis (PCA) and hierarchical clustering of variance-stabilized gene expression across all samples showed that salt stress is the primary driver of transcriptional variation (Fig. 4, Supplementary Fig. 9). PC1 explained 59% of the total variance and clearly separated salt-treated from non-stressed samples. No clear separation was observed for E44 volatile exposure, as the P-buffer control and E44-treated seeds largely overlapped under both conditions (Fig. 4). Similarly, hierarchical clustering produced two main clusters with all salt stress-treated samples grouped together and clearly separated from non-stressed seeds, with no distinct clustering associated with E44 exposure (Supplementary Fig. 9). Together, these results indicate that salt-responsive

transcriptional changes dominate the global transcriptome, whereas E44-responsive transcriptional changes are limited.

To further investigate the role of E44 VOCs during seed germination under salt stress, we performed differential expression analysis. Consistent with the global transcriptomic patterns, this analysis confirmed that salt stress was the dominant factor shaping the germination transcriptome, whereas E44 volatile exposure induced relatively few transcriptional changes (Table 1; Supplementary Table 1). In the absence of E44, salt stress induced 2,342 differentially expressed genes (DEGs), including 972 up-regulated and 1,370 down-regulated genes. A similarly strong salt-responsive transcriptional change was observed in the presence of E44, with 1,754 DEGs (810 up-regulated and 944 down-regulated). In contrast, E44-responsive transcriptional changes were minimal, with only 23 DEGs under non-stress conditions (buffer_Demi vs E44_Demi) and 14 DEGs under salt stress (buffer_NaCl vs E44_NaCl). Together, these results confirm that salinity is the primary driver of transcriptional reprogramming in germinating seeds, while E44 volatiles have a limited direct effect on global gene expression. Notably, the reduced number of salt-responsive DEGs in the presence of E44 suggests that E44 modulates the salt-responsive transcriptional change rather than broadly reprogramming gene expression.

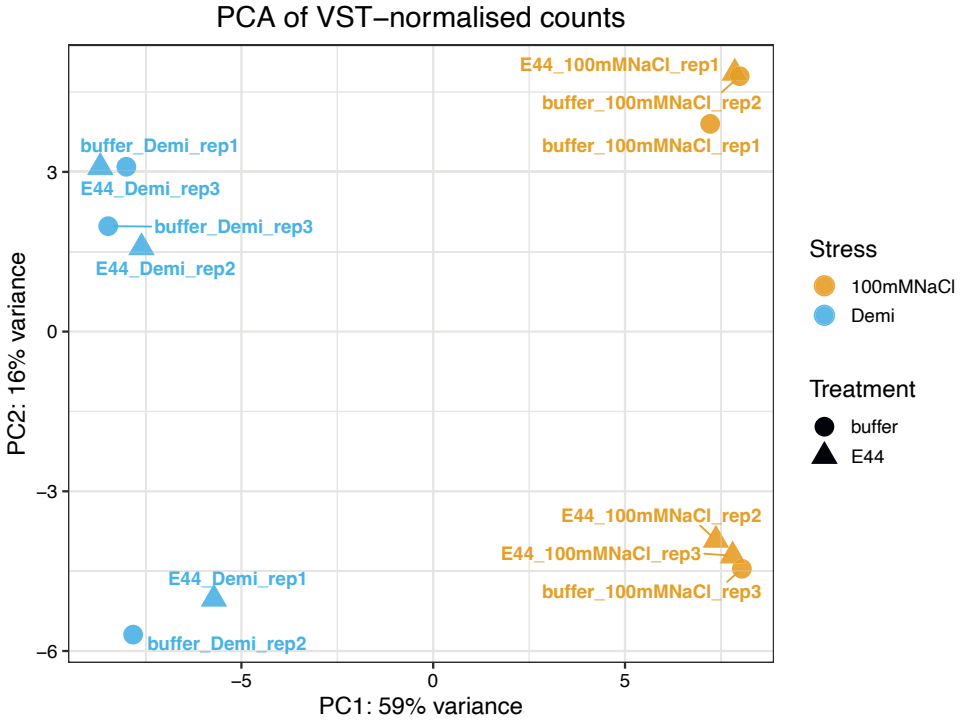


Figure 4: Salt stress is the dominant driver of transcriptional variation in imbibed Arabidopsis seeds, whereas E44 volatiles induce only minor transcriptomic changes. Principal component analysis (PCA) of variance-stabilized gene expression in imbibed *Arabidopsis thaliana* (L*er*) seeds exposed to *Pantoea agglomerans* E44 volatiles or control buffer under salt (100 mM NaCl, imbibed 48 hours) and non-stress (demineralized water, imbibed 30 hours) conditions. Samples are coloured by stress (orange = salt, blue = non-stress) and shaped by treatment (circle = buffer, triangle = E44 volatile). PC1 and PC2 explain 59% and 16% of the variance, respectively. Replicates cluster primarily by stress condition, with clear separation between salt and non-stress samples, while the effect of E44 volatiles is subtler within each stress condition.

Table 1: Number of differentially expressed genes (DEGs) in imbibed *Arabidopsis thaliana* (L*er*) seeds under control and salinity stress with or without E44 volatile exposure. DEGs were identified using DESeq2 with adjusted p-value < 0.05 and $|\log_2$ fold change$|$ > 0.58. Up-regulated genes indicate higher expression in the first condition listed in each contrast, while down-regulated genes indicate lower expression relative to that condition.

Contrast	Experimental comparison	Experimental effect	DEGs		
			Total DEGs	Up-regulated genes	Down-regulated genes
Buffer_Demi vs E44_Demi	Control (P-buffer) vs. E44 Treatment (under non-stress)	E44 effect under non-stress	23	3	20
Buffer_NaCl vs E44_NaCl	Control (P-buffer) vs. E44 Treatment (under salt stress)	E44 effect under salt	14	9	5
Buffer_Demi vs Buffer_NaCl	Non-stressed vs. Salt Stress (in P-buffer control)	Salt effect without E44	2342	972	1370
E44_Demi vs E44_NaCl	Non-stressed vs. Salt Stress (in E44 treatment)	Salt effect with E44	1754	810	944

E44 volatiles modulate the transcriptional response to salt stress in germinating seeds

To distinguish E44-responsive transcriptional changes from salt-responsive responses, we visualized DEGs across all treatments using a Venn diagram. Despite the small number of genes directly responsive to E44 volatiles, phenotypic analyses revealed clear improvements in germination and early seedling growth under salt stress in the L*er* accession (Fig. 1; Supplementary Fig. 2). This suggests that E44 may influence plant performance through targeted modulation of specific pathways rather than broad transcriptional reprogramming. Consistent with this, we found that exposure to E44 volatiles triggered limited and highly specific E44-responsive transcriptional changes, affecting only a small subset of genes. Under non-stress conditions, only a single gene (AT2G34790) was differentially expressed (buffer_Demi vs E44_Demi), whereas five genes were E44-responsive

under salt stress (buffer_NaCl vs E44_NaCl), including AT2G22730, AT1G53230, AT3G59320, AT3G59330, and AT1G26440 (Fig. 5; Table 2). In contrast, salt stress induced extensive transcriptional changes, with 1,108 genes uniquely salt-responsive in the absence of E44 (Buffer_Demi vs Buffer_NaCl) and 1,211 genes commonly regulated under salt stress regardless of E44 exposure.

We next examined whether E44 modulates salt-responsive transcriptional programs by focusing on 532 genes uniquely responsive to salt stress in the presence of E44 (E44_Demi vs E44_NaCl), representing salt-responsive transcriptional changes under E44 exposure (Fig. 5; Table 2 and Supplementary Table 2). The distribution of $\log_2$ fold-change values for these genes showed both positive and negative shifts, indicating the presence of both up- and down-regulated genes (Supplementary Fig. 10). Most genes exhibited moderate changes in expression, although a subset showed stronger responses (|LFC|>2), reflecting pronounced salt-induced transcriptional changes under E44 exposure. Gene Ontology (GO) enrichment analysis was performed to characterize the biological processes associated with these genes. Among the 300 up-regulated genes (Supplementary Table 3), the most enriched categories included secondary metabolic process (n = 23), sulfur compound metabolic process (n = 19), and response to oxidative stress (n = 12). Representative genes within these categories included *Glutathione S-transferase Tau 12* (*ATGSTU12*), β-*Glucosidase 27* (*BGLU27*), *Arabidopsis NAC domain-containing protein 42* (*ANAC042/JUB1*), *MYB domain protein 51* (*AtMYB51*), *Peroxiredoxin IID* (*PRXIID*), and *Peroxidase 4* (*PER4*). In addition, processes related to cellular responses to hypoxia and wounding were also enriched. In contrast, the 232 down-regulated genes were primarily enriched in photosynthesis-related cellular components, including the photosynthetic membrane (n = 10), chloroplast thylakoid membrane (n = 9), plastid thylakoid membrane (n = 9), and thylakoid membrane (n = 9). Additional enrichment was observed for outer membrane (n = 10) and organelle outer membrane (n = 10) (Supplementary Table 4). Representative genes included *Photosystem II reaction center protein A* (*PSBA*), *Photosystem II reaction center protein D* (*PSBD*), *Light-Harvesting Complex Photosystem I Subunit A2* (*LHCA2*), *Photosystem I Subunit O* (*PSAO*), and *Chlororespiratory Reduction 3* (*CRR3*). Together, these results indicate that direct E44-responsive transcriptional changes are limited, whereas E44 markedly reshapes the salt-responsive transcriptional program by enhancing stress-associated pathways and suppressing photosynthesis-related processes. This shift suggests that E44 promotes a more stress-adaptive transcriptional state in germinating seeds under salinity.

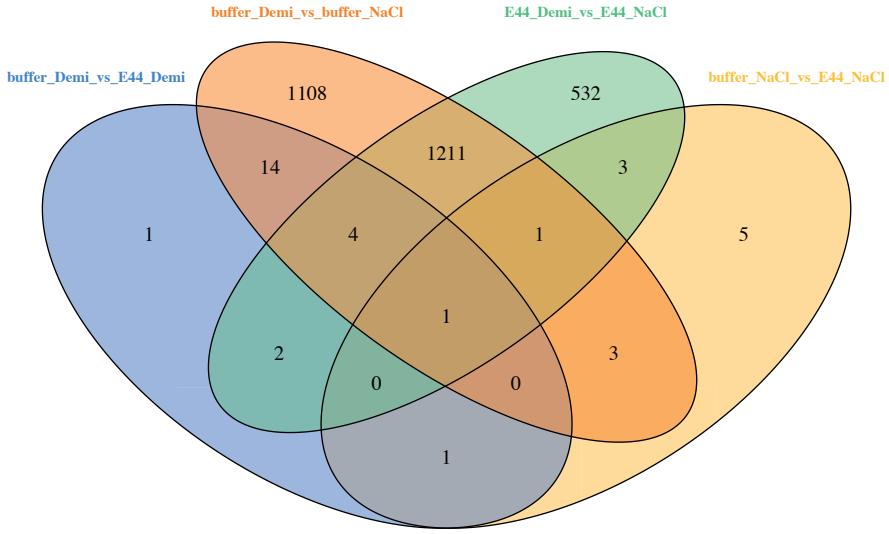


Figure 5: E44 volatiles reshape the salt-responsive transcriptional response in imbibed Arabidopsis seeds. Four-way Venn diagram showing the number of differentially expressed genes (DEGs) identified across four pairwise comparisons: buffer_Demi vs. buffer_NaCl (salt stress without bacterial volatiles), E44_Demi vs. E44_NaCl (salt stress in the presence of E44 volatiles), buffer_Demi vs. E44_Demi (effect of E44 volatiles under non-stress conditions), and buffer_NaCl vs. E44_NaCl (effect of E44 volatiles under salt stress). Imbibed seeds of the *Arabidopsis thaliana* accession L*er* were exposed to either E44 bacterial volatiles or P-buffer (non-bacterial volatile control) under non-stress conditions (demineralized water) or salt stress (100 mM NaCl). Differentially expressed genes were identified using DESeq2 with an adjusted p-value < 0.05 and an absolute $\log_2$ fold change > 0.58. Numbers indicate the total number of DEGs in each comparison regardless of direction of regulation, and overlapping regions represent genes shared between contrasts.

Table 2: Classification of differentially expressed genes (DEGs) based on E44-induced and salt-induced transcriptional changes in imbibed *Arabidopsis thaliana* (L*er*) seeds. DEGs (adjusted p-value < 0.05, $|\log_2$ fold change$|$ > 0.58) are grouped based on overlap across four contrasts: buffer_Demi vs E44_Demi, buffer_NaCl vs E44_NaCl, buffer_Demi vs buffer_NaCl, and E44_Demi vs E44_NaCl. Key genes are highlighted for each category, and full DEG lists for larger groups are provided in Supplementary Table 2. Categories represent unique and shared (indicated by "overlap") and transcriptional responses to E44 volatile exposure and salt stress, with overlaps corresponding to intersections shown in the Venn diagram (Fig. 5).

Category	Contrast	DEGs (n)	Key Genes
E44-responsive (non-stress)	buffer_Demi vs E44_Demi	1	AT2G34790 (FAD-binding Berberine family protein)
E44-responsive (salt stress)	buffer_NaCl vs E44_NaCl	5	AT2G22730 (Major facilitator superfamily) AT1G53230 (TEOSINTE BRANCHED 1) AT3G59320 (solute carrier family 35 protein (DUF914) AT3G59330 (Solute carrier family 35 DUF914) AT1G26440 (Ureide permease 5)
Salt-responsive (without E44)	buffer_Demi vs buffer_NaCl	1,108	(See Supplementary Table 2)
Salt-responsive (with E44)	E44_Demi vs E44_NaCl	532	(See Supplementary Table 2)
Shared salt-responsive (independent of E44)	Overlap between buffer_Demi vs buffer_NaCl and E44_Demi vs E44_ NaCl	1,211	(See Supplementary Table 2)
Shared E44-responsive (non-stress) and salt-responsive (without E44)	Overlap between buffer_Demi vs E44_ Demi and buffer_Demi vs buffer_NaCl	14	AT1G10155 : phloem protein 2-A10 AT1G12240 : Glycosyl hydrolases family 32 protein AT1G18880 : Major facilitator superfamily protein AT1G23720 : Proline-rich extensin-like family protein AT2G27140 : HSP20-like chaperones superfamily protein AT2G39310 : jacalin-related lectin 22 AT3G28550 : Proline-rich extensin-like family protein AT4G04750 : Major facilitator superfamily protein AT4G27440 : protochlorophyllide oxidoreductase B AT4G30640 : RNI-like superfamily protein AT5G15850 : CONSTANS-like 1 AT5G17330 : glutamate decarboxylase AT5G43300 : PLC-like phosphodiesterases superfamily protein AT5G67390 : glycosyltransferase-like protein

Table 2: Continued

Category	Contrast	DEGs (n)	Key Genes
Shared E44-responsive (salt stress) and salt-responsive (with E44)	Overlap between buffer_NaCl vs E44_NaCl and E44_Demi vs E44_NaCl	3	AT4G11170 : Disease resistance protein (TIR-NBS-LRR class) family AT4G16857 : disease resistance protein (TIR-NBS-LRR class) family protein AT5G57550 : xyloglucan endotransglucosylase/hydrolase 25
Shared across all conditions (E44- and salt-responsive)	Intersection of all four contrasts	1	AT1G56580 (Protein of unknown function, DUF538)

E44 volatiles mitigated salt-responsive transcriptional changes

Following the identification of 532 genes uniquely differentially expressed in response to salt stress in the presence of E44 volatiles, we next examined whether E44 affects the overall transcriptional response to salinity. To address this, we compared salt-responsive gene expression in the absence (buffer_Demi vs buffer_NaCl) and presence (E44_Demi vs E44_NaCl) of E44 volatiles across all expressed genes. By plotting the $\log_2$ fold-change (LFC) values for each gene across both conditions (Fig. 6; Supplementary Table 5), we assessed how E44 exposure influences salt-responsive transcriptional programs. In this scatter plot, the x-axis represents the transcriptional response to salt stress in the absence of E44 volatiles, whereas the y-axis represents the corresponding response in the presence of E44. Most genes clustered along the diagonal, indicating that salt-responsive expression patterns were broadly similar under both conditions, while genes deviating from the diagonal reflect E44-associated differences in the salt response. The majority of genes (20,837), predominantly non-significant genes (grey points), were located near the center of the plot, indicating little or no salt responsiveness regardless of E44 exposure. Consistently, genes significantly responsive to salt stress in both conditions (salt-responsive genes; SRGs; orange points; 1,217 genes) showed similar fold-change values in the presence and absence of E44, indicating that the core salt stress response remained largely conserved. These genes included both upregulated genes in the upper-right quadrant and downregulated genes in the lower-left quadrant, indicating that they responded to salt stress in the same direction under both treatments. This shows that the core salt stress response remained largely similar with or without E44 exposure. Moreover, a very small number of SRGs were observed in the lower-right quadrant, exhibiting opposing fold-change values between conditions (positive in the absence of E44 and negative in its presence), indicating that the direction of salt-responsive regulation can differ under E44 exposure. This suggests that E44 can alter the transcriptional behavior of a limited subset of genes, switching their regulation from activation to repression; however, this represented only a minor fraction of the overall response.

Notably, a large group of genes clustered near the horizontal axis ($y \approx 0$), corresponding to genes that were salt-responsive only in the absence of E44 and lost statistical significance in its presence. These genes represent “rescued” transcripts (1,125 genes; green points), for which salt-responsive transcriptional changes were reduced or eliminated by E44 exposure. This effect was observed for both down-regulated ($x < 0$) and up-regulated ($x > 0$) that were salt-responsive in the absence of E44, which shifted toward $y \approx 0$ in the presence of E44, indicating reduced salt responsiveness. A small subset of these rescued

genes, located in the lower-right quadrant, displayed strong positive fold-change values in the absence of E44 but negative values in its presence. However, these genes were not classified as salt-responsive under E44 conditions because their expression changes did not meet the statistical significance threshold, indicating that their responses were not robustly maintained in the presence of E44 alone. In addition, a smaller group of genes (537 genes; blue points) was significantly salt-responsive only in the presence of E44 volatiles, indicating that E44 enables condition-specific salt-responsive transcriptional changes in a limited subset of genes. These genes were distributed around the vertical axis ($x \approx 0$), reflecting the lack of significant salt responsiveness in the absence of E44. Together, these results show that the core salt-responsive transcriptional program remains largely preserved in the presence of E44 volatiles, while E44 reduces the strength of this response in a substantial subset of genes. The full list of genes is provided in Supplementary Table 5.

To further characterize the rescued genes and those specifically responsive to salt stress in the presence of E44 volatiles, Gene Ontology (GO) enrichment analysis was performed. We found that the 537 genes specifically responsive to salt stress only under E44 exposure (blue points; Fig. 6) corresponded closely to the E44-specific gene set (Fig. 5). This set comprised 532 genes unique to the E44_Demi vs E44_NaCl comparison, together with three genes shared with the buffer_NaCl vs E44_NaCl comparison and two genes shared with the buffer_Demi vs E44_Demi comparison (Supplementary Fig. 11). These genes represent the 537 E44-specific salt-responsive genes identified in Fig. 6, demonstrating strong agreement between the two analytical approaches. We next focused on the 1,125 rescued genes, of which 1,057 were associated with GO annotations and included in the analysis (Table 3; Supplementary Table 6). The most enriched biological processes included ribosome biogenesis (55 genes), ribonucleoprotein complex biogenesis (59 genes), rRNA metabolic process (41 genes), and rRNA processing (39 genes). Enriched cellular component terms included cytosolic ribosome (51 genes), ribosomal subunit (49 genes), and ribosome (53 genes), while molecular function terms were dominated by structural constituent of ribosome (37 genes) and structural molecule activity (41 genes). These results indicate that many genes with reduced or eliminated salt responsiveness in the presence of E44 are involved in ribosome assembly and protein synthesis. Representative genes within these categories included *Nucleolin 1* (*ATNUC-L1*), *Enhancer of Brx1 Protein 2* (*EBP2*), *Nucleolar Complex Protein 2* (*AtNOC2*), *Peter Pan* (*PPAN*), and *Ribosomal Protein L16* (*RPL16*). These results indicate that E44 exposure mitigates salt responsiveness in a substantial subset of genes, particularly those involved in ribosome biogenesis and protein synthesis, while also enabling a smaller set of E44 stress responsive genes.

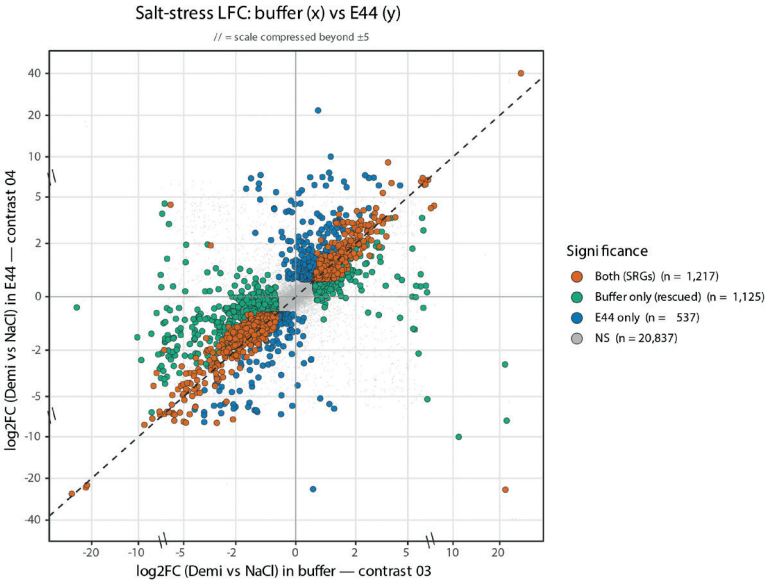


Figure 6: E44 volatiles partially mitigate salt stress-induced transcriptional responses in imbibed *Arabidopsis thaliana* seeds. Scatter plot comparing salt-induced gene expression changes (log_2 fold change, LFC) in imbibed *Arabidopsis thaliana* (L*er*) seeds exposed to a non-bacterial volatile control (P-buffer) or *Pantoea agglomerans* E44 volatiles under 100 mM NaCl. The x-axis represents salt-induced expression changes in the non-bacterial volatile control (buffer_Demi vs buffer_NaCl), while the y-axis represents salt-induced expression changes in the presence of *Pantoea agglomerans* E44 volatiles (E44_Demi vs E44_NaCl). Each point corresponds to a gene. The dashed diagonal line indicates identical transcriptional responses to salt stress under both conditions. Genes significantly responsive to salt stress in both treatments are shown in orange points (salt-responsive genes, SRGs). Genes significantly responsive only in the buffer control are shown in green points ("rescued" by E44), whereas genes significant only in the E44 treatment are shown in blue points. Grey points represent genes that are not significantly differentially expressed.

Table 3: Gene Ontology (GO) enrichment analysis of the rescued genes. The table shows the top significantly enriched GO terms, categorized into biological process (BP), cellular component (CC), and molecular function (MF), with gene counts ranging from 59 to 37. These enriched terms are primarily related to ribosome biogenesis, rRNA processing, and protein synthesis. The full list of enriched GO terms is provided in Supplementary Table 6.

ID	Ontology	Description	p-value	Representative genes	Gene count
GO:0022613	BP	ribonucleoprotein complex biogenesis	1.69×10^{-17}	ATNUC-L1, EBP2, AtNOC2, PPAN	59
GO:0042254	BP	ribosome biogenesis	6.34×10^{-19}	ATNUC-L1, EBP2, AtNOC2, PPAN	55
GO:0005840	CC	ribosome	3.83×10^{-14}	AtTGG1, KCS1, RPL16	53
GO:0022626	CC	cytosolic ribosome	7.32×10^{-18}	AtTGG1, KCS1, RPL16	51
GO:0044391	CC	ribosomal subunit	1.67×10^{-15}	RPL16	49
GO:0034470	BP	ncRNA processing	1.35×10^{-10}	ATNUC-L1, EBP2, PPAN	46
GO:0016072	BP	rRNA metabolic process	5.44×10^{-14}	ATNUC-L1, EBP2, PPAN	41
GO:0005198	MF	structural molecule activity	5.06×10^{-6}	RPL16	41
GO:0006364	BP	rRNA processing	7.12×10^{-14}	ATNUC-L1, EBP2, PPAN	39
GO:0003735	MF	structural constituent of ribosome	1.35×10^{-8}	RPL16	37

DISCUSSION

Microbial volatile organic compounds (VOCs) are increasingly recognised as long- and short-distance signalling molecules in plant-microbe interactions capable of modulating plant growth, defence and responses to environmental stress without direct microbial contact (Fincheira et al., 2021). In this study, we demonstrated that volatiles produced by the seed bacterial endophyte *Pantoea agglomerans* E44, isolated from wild cabbage, enhance *Arabidopsis thaliana* seed germination, seedling biomass, and root system development under abiotic stress conditions, particularly salinity and osmotic stress. These effects were strongly genotype-dependent, with accessions such as L*er*, Bay, and Sha showing more pronounced responses than Col-0, highlighting the importance of host genetic background in determining responsiveness to bacterial volatiles. At the transcriptional level, RNA-seq analysis of imbibed seeds prior to radicle emergence showed that salinity was the main driver of global gene expression changes, reflecting its strong physiological impact during early germination. In contrast, direct responses to E44 volatiles were limited, with only a few differentially expressed genes detected under either control or saline conditions. However, E44 substantially altered the salt-responsive transcriptional program. It induced a distinct set of salt-responsive genes enriched in stress adaptation, and mitigated the expression changes of many salt-responsive genes associated with ribosome biogenesis, rRNA processing, and protein synthesis. These findings suggest that E44 volatiles do not broadly reprogram seed gene expression but instead fine-tune stress-responsive transcriptional networks. Taken together, the results indicate that E44 volatiles enhance early plant development under abiotic stress by promoting adaptive stress responses while reducing salinity-induced disruption of growth-related cellular processes.

Pantoea agglomerans is a widely distributed seed-associated bacterium found in a variety of plant species, including onion (Moloto et al., 2020), wheat (Soluch et al., 2021) and cabbage (Tyc et al., 2020). It is known for its plant growth-promoting potential and its ability to suppress plant pathogens (Sanz-Puente et al., 2025). Many studies on *P. agglomerans* have primarily focused on its role in plant pathogen control through direct inoculation (Duchateau et al., 2024; Hsieh et al., 2005; Lee et al., 2023), while relatively little attention was given to the effects of its volatile compounds, particularly under abiotic stress conditions. Previously, Tyc et al. (2020) isolated *P. agglomerans* E44 from wild cabbage seeds and found that its volatile compounds promoted the seed germination and seedling primary root length of cabbage under non-stress conditions compared to volatiles emitted by other seed-associated bacterial endophytes. Notably, the *P. agglomerans* E44 strain used in our study is identical to the strain described by

Tyc et al. (2020), enabling a direct comparison of VOC-mediated effects under different environmental conditions. While those findings demonstrated growth-promoting effects under non-stress conditions, our results (Figs. 1-3) show that E44-derived VOCs significantly enhanced seed germination and seedling growth under salinity and osmotic stress, with little to no effect under non-stress conditions. Together, these findings suggest that the beneficial effects of E44 volatiles are conserved across Brassicaceae species, including cabbage and *Arabidopsis thaliana*, and occur mainly under abiotic stress rather than non-stress conditions, supporting a role for E44-derived VOCs in enhancing stress resilience.

Evidence supporting the role of *Pantoea agglomerans* VOCs in abiotic stress responses remains limited. However, VOCs from *P. agglomerans* MVC21 were previously shown to promote root and shoot growth in tomato seedlings under non-stress conditions, with dimethyl disulfide (DMDS) producing effects similar to the full VOC blend (Vasseur-Coronado et al., 2021). Sulfur-containing compounds such as DMDS and dimethyl trisulfide (DMTS) have also been consistently detected in our candidate strain, *P. agglomerans* E44, and may represent important components of its VOC profile (Tyc et al., 2020). Several studies have shown that DMDS, a compound produced by many plant growth-promoting bacteria and often considered a key bioactive component of microbial VOC blends, can promote plant growth and abiotic stress tolerance by enhancing germination, modifying root architecture, increasing biomass, and reducing oxidative stress (Lin et al., 2025; Luo et al., 2022; Türksoy et al., 2025). These effects are typically mediated by VOC blends rather than single compounds, indicating that interactions among multiple bacterial volatiles likely shape plant growth, root development, and stress responses. DMDS has also been linked to auxin-related signalling, leading to reduced primary root elongation and increased lateral root formation (Tyagi et al., 2019). This is consistent with our results, where E44 volatiles increased lateral root number while reducing primary root length in some Arabidopsis accessions (Fig. 3). Together, these findings suggest that DMDS, previously identified as part of the VOC profile of *P. agglomerans* E44, may contribute to the enhanced germination and seedling growth observed under salinity and osmotic stress in our study. However, bacterial VOC production is highly dependent on environmental and experimental conditions, and the presence or abundance of DMDS under our specific assay conditions was not determined. Therefore, the observed effects are more likely mediated by the overall VOC blend emitted by E44 rather than by a single compound. Future studies should characterise the VOC profile of E44 under our experimental conditions, including quantification of DMDS, and test purified compounds individually and in combination to determine their specific and dose-dependent roles.

While specific VOCs such as DMDS can modulate plant hormonal pathways, the level of these responses appears to depend strongly on plant genotype, as observed in this study where responses to *Pantoea agglomerans* E44 volatiles varied among Arabidopsis accessions (Figs. 1–3). In particular, the accessions L*er*, Bay, and Sha showed improvements in germination, seedling growth, and root development under stress conditions, whereas Col-0 was less responsive or even negatively affected under the tested conditions. Such genotype-dependent responses to microbial volatiles have been reported previously. Wintermans et al. (2016) showed that Arabidopsis exhibits natural variation in its response to rhizobacteria, including VOC-related effects, reflecting differences in microbial signal perception and associated processes such as hormonal regulation and root development. These findings suggest that variation in plant responses is not only driven by the presence of specific VOCs but also by differences in how plant genotypes perceive and integrate these signals. This variation is likely linked to differences in underlying signalling pathways, as plant responses to microbial VOCs are mediated by complex networks involving hormones such as auxin, abscisic acid, jasmonic acid, and ethylene, as well as stress-related transcriptional regulation (Cordovez et al., 2017; Kanjana et al., 2026; Sharifi & Ryu, 2018). In addition, *P. agglomerans* can exhibit both beneficial and pathogenic effects depending on the plant species and environmental conditions (Dutkiewicz et al., 2016; Walterson & Stavrinides, 2015). This dual potential may contribute to the variation observed among Arabidopsis accessions in our study, highlighting the importance of host-microbe specificity in shaping plant responses to microbial VOCs. Future studies should evaluate the effects of E44 on its original host species under stress conditions to better understand the ecological relevance of this interaction. In addition, testing VOCs from bacterial strains isolated directly from Arabidopsis may help determine whether native plant-microbe associations result in stronger or more consistent growth-promoting effects. Overall, these findings suggest that the effectiveness of microbial VOCs depends on both plant genotype and environmental conditions, which should be considered when developing applications for agricultural systems.

In addition to the positive effects of *P. agglomerans* E44 volatiles, our results showed that volatiles emitted by *E. coli* DH5α, a non-typical plant-associated bacterium, also promoted seed germination and root growth in some Arabidopsis accessions (Figs. 1–3). *E. coli* DH5α is known to produce several bioactive VOCs, including indole, ketones, alcohols, and 2-phenylethanol, which can influence plant physiology and root development through hormonal pathways such as auxin signalling (Almeida et al., 2022; Bailly et al., 2014; Eshima et al., 2025). Previous studies have shown that these VOCs can enhance biomass accumulation and root development in rice and Arabidopsis (Almeida et al., 2022; Bailly et al.,

2014). However, compared with plant-associated bacteria, the volatile profile of *E. coli* is less complex and likely less specialised for plant interaction (Eshima et al., 2025). This is consistent with our results, where the effects of E44 volatiles were generally stronger and more consistent across accessions and stress conditions than those of *E. coli*, suggesting that E44 produces a more effective VOC blend for modulating plant growth under stress.

While *P. agglomerans* E44 volatiles clearly have a positive effect on early plant development under abiotic stress conditions, it remains unclear to what extent these phenotypic effects are reflected at the transcriptional level. Our transcriptomic analysis showed that salt stress was the dominant factor shaping gene expression in imbibed Arabidopsis seeds (Table 1), inducing extensive transcriptional reprogramming, consistent with the established role of salinity in driving extensive transcriptional change during early plant development (Zhang et al., 2019). It should be noted that non-stressed and salt-stressed seeds were sampled at different times after imbibition (30 h and 48 h, respectively). This sampling strategy was intentionally conducted to compare seeds at a similar physiological stage prior to radicle emergence, as salinity substantially delayed germination. Nevertheless, we cannot exclude the possibility that some transcriptional differences are related to the longer imbibition period of salt-treated seeds, rather than being caused exclusively by salinity. Despite this limitation, many salt-responsive genes were shared across treatments, indicating that most transcriptional responses to salinity were largely independent of bacterial volatile exposure.

E44 volatile exposure triggered only a limited and highly specific response, with few E44-responsive genes and minimal overlap with salt-responsive genes (Table 1; Fig. 5; Table 2). Notably, these direct E44 responses were strongly condition dependent. Under non-stress conditions, only a single gene responded to E44, suggesting little transcriptional impact when environmental conditions were favourable. Under salinity, however, five genes were specifically E44-responsive, indicating that E44 still leads to small transcriptional changes under stress conditions (Table 2). Among these five genes, *UREIDE PERMEASE 5* (*UPS5*; AT1G26440) was the only gene with a well-established role in salt stress responses. UPS5 is associated with protection against osmotic stress, ROS detoxification, and stress signalling, and mutants lacking UPS5 show increased salt sensitivity (Lescano et al., 2016). The E44-mediated induction of *UPS5* therefore suggests that bacterial volatiles may enhance protective pathways supporting germination under salinity. These findings suggest that beneficial effects of E44 are mediated through targeted regulatory processes rather than broad transcriptional reprogramming. This is consistent with previous studies

showing that microbial volatiles induce the expression of relatively small and targeted gene sets associated with developmental signalling pathways, including auxin and cytokinin signalling, rather than triggering global transcriptional reprogramming (Chang et al., 2024; Tao et al., 2026).

E44 volatiles clearly altered the transcriptional response of germinating seeds to salinity, as shown by the 532 genes that were uniquely salt responsive in the presence of E44, indicating a distinct transcriptional programme under salt stress (Fig. 5; Table 2). Functional enrichment of upregulated genes suggests that E44 stimulates stress-adaptive processes related to redox balance, detoxification, and metabolic adjustment (Supplementary Table 3). Representative genes identified in our study further support stress-adaptive functions. *ATGSTU12* is linked to detoxification and antioxidant defence (Nguyen et al., 2017), while *ATC4H*, a key enzyme in the phenylpropanoid pathway, suggests enhanced secondary metabolism associated with antioxidant and structural protection (Kumar et al., 2016; Rostami et al., 2022). The genes *AtMYB51* and *ANAC042/JUB1*, which encode transcription factors, are associated with abiotic stress-responsive signalling and salinity tolerance in Arabidopsis (Roy, 2016; Wu et al., 2012). In contrast, genes downregulated under E44 exposure were mainly associated with photosynthetic and chloroplast functions (Supplementary Table 4), suggesting reduced investment in photosynthesis under salinity stress. Representative genes included *PSBA*, *PSBD*, and *LHCA2*, which encode core components of photosystem II and the light-harvesting complex of photosystem I (Dreyfuss & Thornber, 1994; Millard et al., 2004). Their coordinated downregulation is consistent with reduced photosynthetic activity and chloroplast function, often associated with lower chlorophyll content and decreased photosynthetic capacity (Azarin et al., 2020). Temporary suppression of photosynthesis may represent a protective acclimation strategy that limits ROS formation and oxidative stress during early germination, when growth depends largely on stored reserves (Dalal & Tripathy, 2018). Collectively, these results suggest that E44 reshapes the salinity response of germinating seeds by promoting stress-adaptive pathways while simultaneously suppressing energy-demanding photosynthetic functions, thereby supporting early seed performance under salt stress.

The relatively small number of differentially expressed genes in response to E44 volatiles may reflect the early developmental stage analysed, as seeds were sampled during late imbibition prior to radicle emergence, when transcriptional activity remains lower than at later stages of germination (Dekkers et al., 2013; Zhang et al., 2019). Gene regulation during imbibition is not only dependent on transcription but also involves the selective translation of stored mRNAs retained from seed maturation, enabling rapid protein synthesis prior to extensive

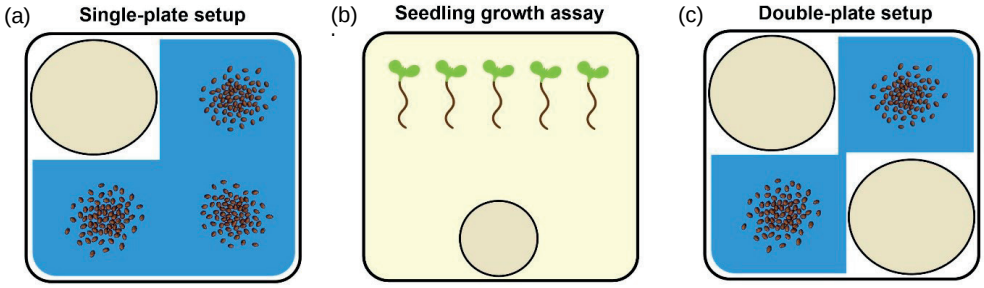
transcriptional activation (Bai et al., 2017). The relatively few transcriptional changes detected despite clear phenotypic effects, suggest that bacterial volatiles can influence processes that are not captured by RNA-seq alone. This point of view finds support by having a closer look at the rescued genes, defined as genes that were significantly salt responsive in the absence of E44 volatiles but no longer differentially regulated when E44 was present (Fig. 6; Supplementary Table 5). Gene Ontology analysis revealed strong enrichment of rescued genes involved in ribosome biogenesis, rRNA processing, and protein synthesis (Table 3; Supplementary Table 6). Representative rescued genes such as *ATNUC-L1*, *AtNOC2*, and *RPL16* further support this pattern, suggesting that E44 helps maintain ribosome biogenesis and translational capacity under salt stress. These processes are important during germination, when protein synthesis is essential. *ATNUC-L1* functions in nucleolar organization and rRNA processing, supporting ribosome biogenesis in Arabidopsis (Pontvianne et al., 2007), *AtNOC2* functions in ribosomal subunit maturation in *Arabidopsis* (de Bossoreille et al., 2018), and *RPL16* encodes a core component of the large ribosomal subunit required for translation and normal growth (Brehme et al., 2015; Li et al., 2023). Their loss of salt responsiveness in the presence of E44 suggests that E44 may help preserve ribosome production, protein synthesis, and growth-related processes during germination under salinity. Abiotic stress often suppresses ribosome biogenesis and translational activity to conserve energy for survival processes (Wu et al., 2023). Therefore, maintenance of these processes under E44 exposure may indicate reduced stress severity and improved germination performance, as translation of stored mRNAs after imbibition is required for metabolic activation and seedling establishment (Galland & Rajjou, 2015; Sajeev et al., 2019).

In conclusion, our study demonstrates that volatiles produced by the seed-associated bacterium *Pantoea agglomerans* E44 enhance Arabidopsis seed germination and early seedling development under abiotic stress conditions, particularly salinity and osmotic stress. These effects are strongly genotype-dependent. Transcriptomic analysis of imbibed seeds revealed that salinity was the primary driver of gene expression changes, whereas direct transcriptional responses to E44 volatiles were limited. The most prominent effect of E44 was the mitigation of salt-induced transcriptional responses. A substantial group of salt-responsive genes (1,125 genes) was no longer significantly regulated in the presence of E44, indicating that E44 mitigated many of the transcriptional changes normally triggered by salinity. These rescued genes were enriched for functions related to ribosome biogenesis, rRNA processing, and protein synthesis, suggesting that E44 may help maintain growth-related cellular processes during salt stress. In addition, E44 induced a smaller set of salt-responsive genes (532 genes) that were enriched in stress adaptation functions, including antioxidant

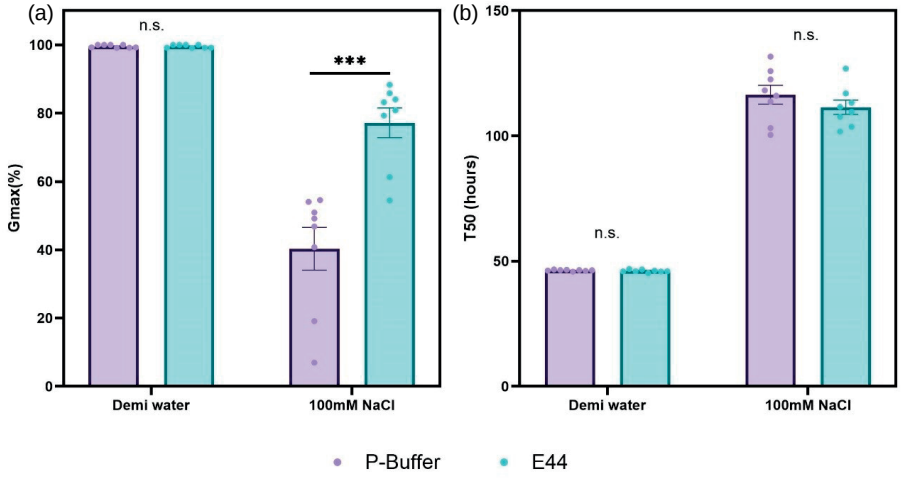
defence, detoxification, and protective stress responses. Together, these findings suggest that E44 volatiles do not broadly reprogram gene expression but instead reshape seed responses to salinity by reducing stress-induced disruption of essential cellular functions while promoting adaptive stress-response pathways. This effect may contribute to the enhanced germination and seedling growth observed under salt and osmotic stress conditions.

Future studies integrating epigenetic, transcriptomic, and translational approaches, combined with reverse-genetic analysis, will be important to better understand the mechanisms underlying VOC-mediated priming. In addition, the application potential of individual VOC compounds from the E44 blend, such as dimethyl disulfide (DMDS), should be explored by testing their effects on seed germination and stress tolerance in both model plants and crop species. It will be important to assess dose-dependent effects and develop practical application strategies relevant to the seed industry, such as seed bio-priming with bacterial VOCs or live E44 inoculants or other VOC-producing beneficial bacteria, as well as seed coating. It would also be valuable to test VOCs produced by seed endophytes isolated directly from Arabidopsis to determine whether native host-microbe associations result in stronger or more consistent effects. Such approaches may provide a basis for developing sustainable, microbe-seed treatments to improve crop resilience under abiotic stress conditions.

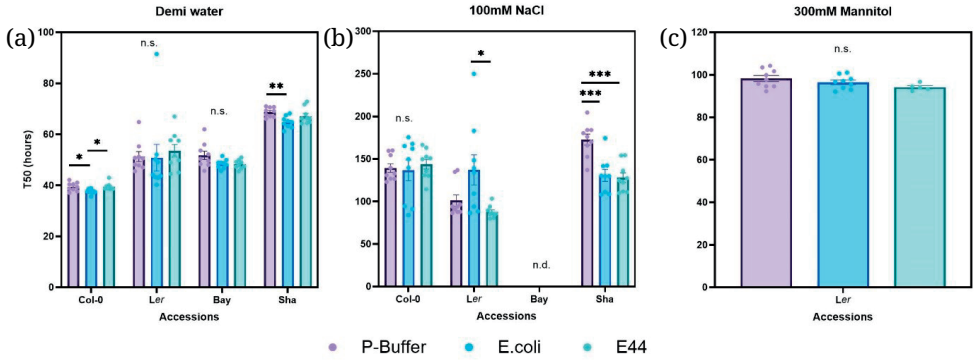
SUPPLEMENTARY FIGURES



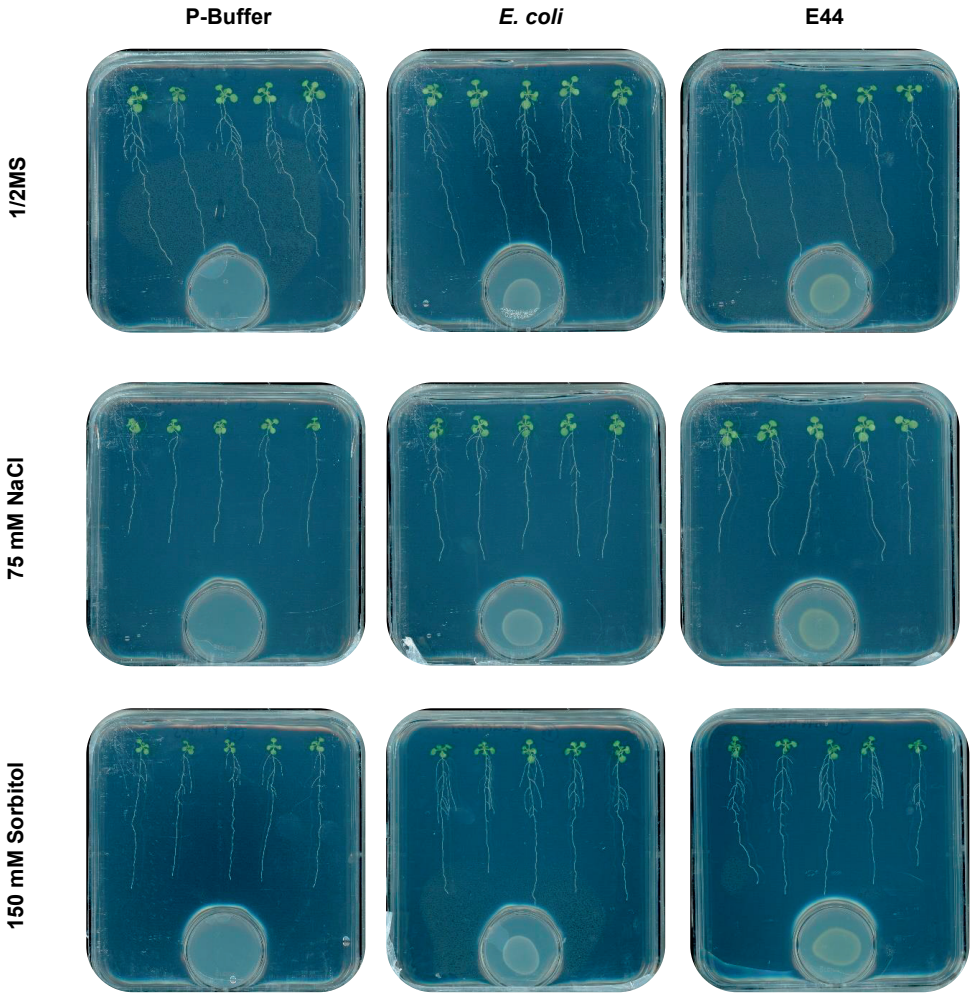
Supplementary Figure 1: Experimental setups for assessing the effects of bacterial volatiles on Arabidopsis. (a) Single-plate setup: Used for the germination assay. One bacterial or buffer control plate was placed in the top-left corner of the germination plate, with three replicates of approximately 50 Arabidopsis seeds each. (b) Seedling growth assay: Used for seedling growth in response to volatiles. A bacterial or buffer control plate was positioned at the bottom center of a ½ MS square plate, with five three-day-old seedlings placed at the top of the plate. (c) Double-plate setup: Used for RNA-seq experiments. Two bacterial or buffer control plates were placed at the top-left and bottom-right corners to generate a higher volatile concentration. In all setups, seeds and seedlings were exposed only to volatiles, without direct contact with the bacteria.



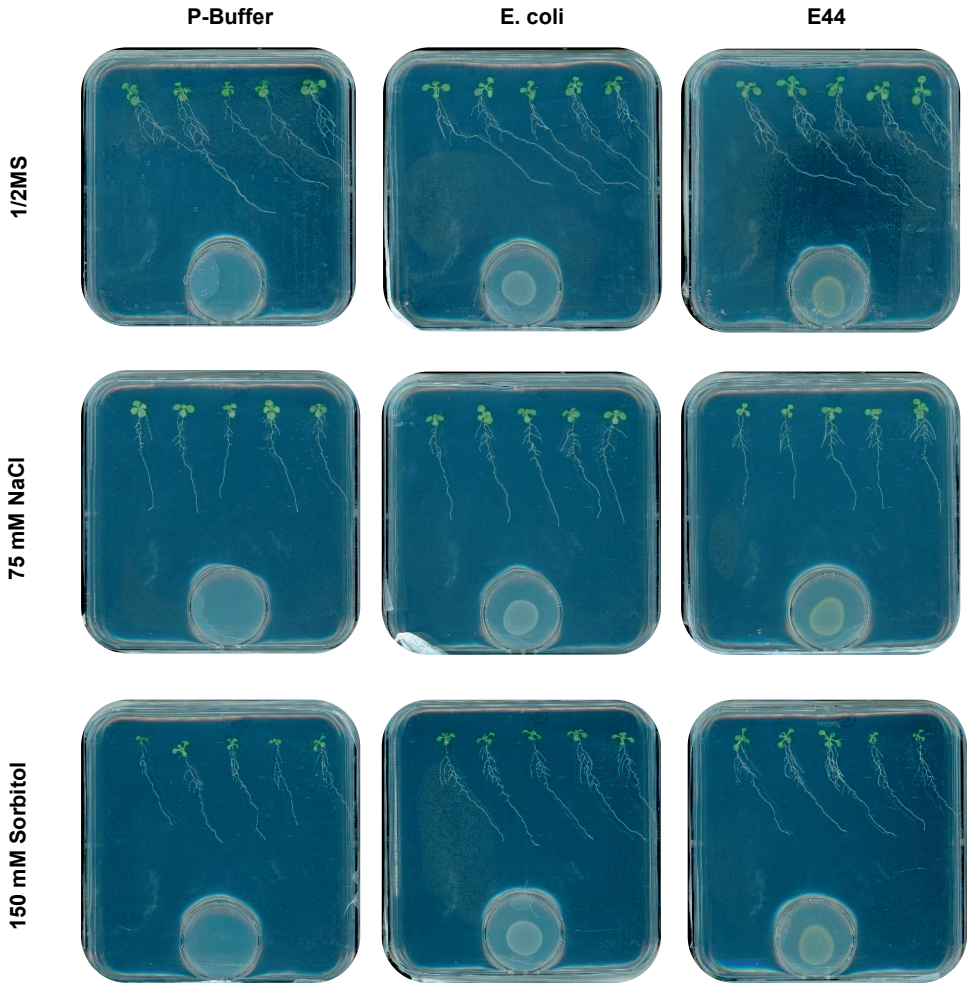
Supplementary Figure 2.: Volatiles from *Pantoea agglomerans* E44 promote Arabidopsis seed germination in a double-plate setup. *Ler* seeds were imbibed in demineralized water (non-stress) or 100 mM NaCl (salinity stress) and exposed to bacterial volatiles without direct contact. Control seeds were exposed to p-buffer plates to account for non-volatile effects. (a) Maximum germination percentage (Gmax). (b) Time to 50% germination (T50). Data represent the mean ± standard error of the mean (SEM) of eight technical replicates per treatment. Statistical comparisons between volatile-exposed and control seeds were performed using Welch's t-test. Statistical significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), n.s., not significant. Exposure to E44 volatiles consistently stimulated germination under salinity stress. Sampling time points for RNA-seq were selected prior to radicle protrusion based on these germination dynamics.



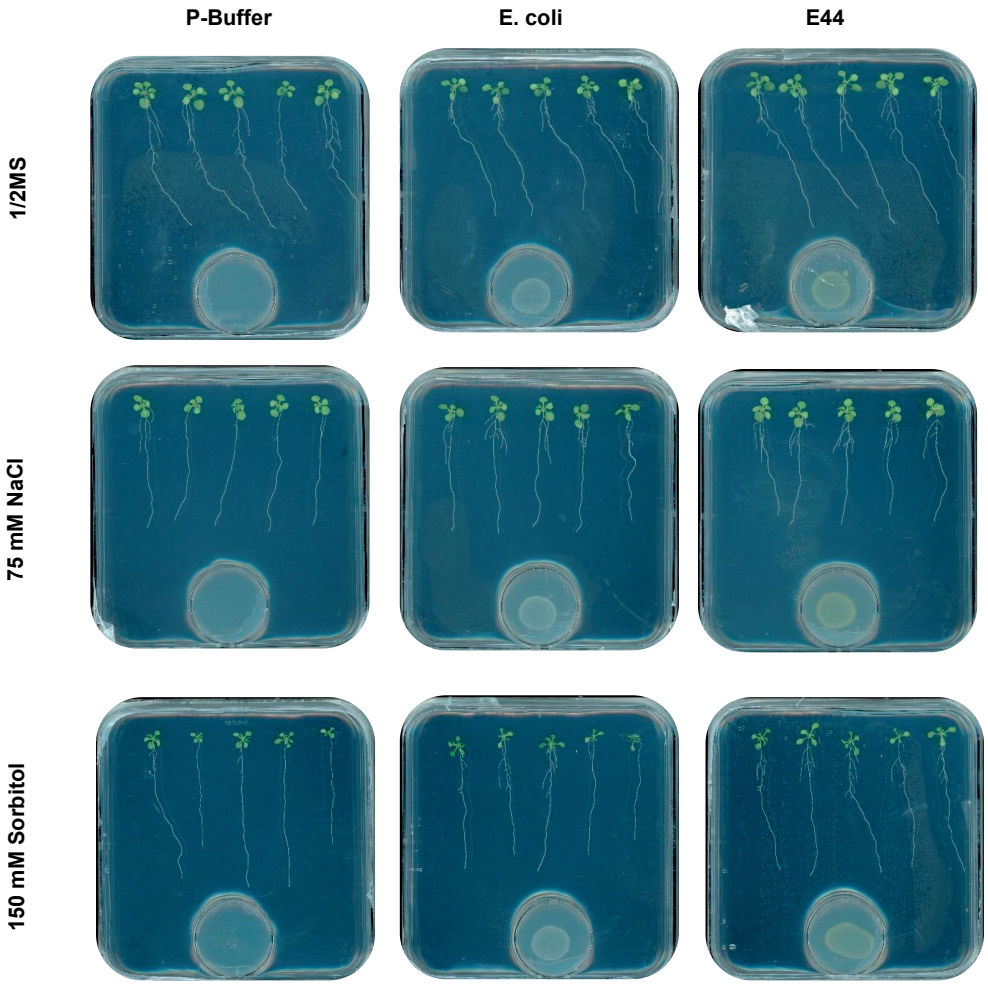
Supplementary Figure 3: Germination rate (T50) of seeds from four *Arabidopsis thaliana* accessions (Col-0, L*er*, Bay, and Sha) in the presence or absence of bacterial volatiles under non-stress and abiotic stress conditions. Seeds were exposed to three treatments: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* (*E. coli*) (positive control), and volatiles produced by *Pantoea agglomerans* E44, a bacterial strain isolated from wild cabbage seeds. T50 was assessed under three conditions: (a) non-stress conditions with demineralized water, (b) salinity stress with 100 mM NaCl, and (c) osmotic stress with 300 mM mannitol. Data are shown as mean ± standard error of the mean (SEM). Statistical differences among volatile treatments within each accession were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, n.s., not significant., and n.d., not determined, used for T50 when germination was too low to calculate reliably.



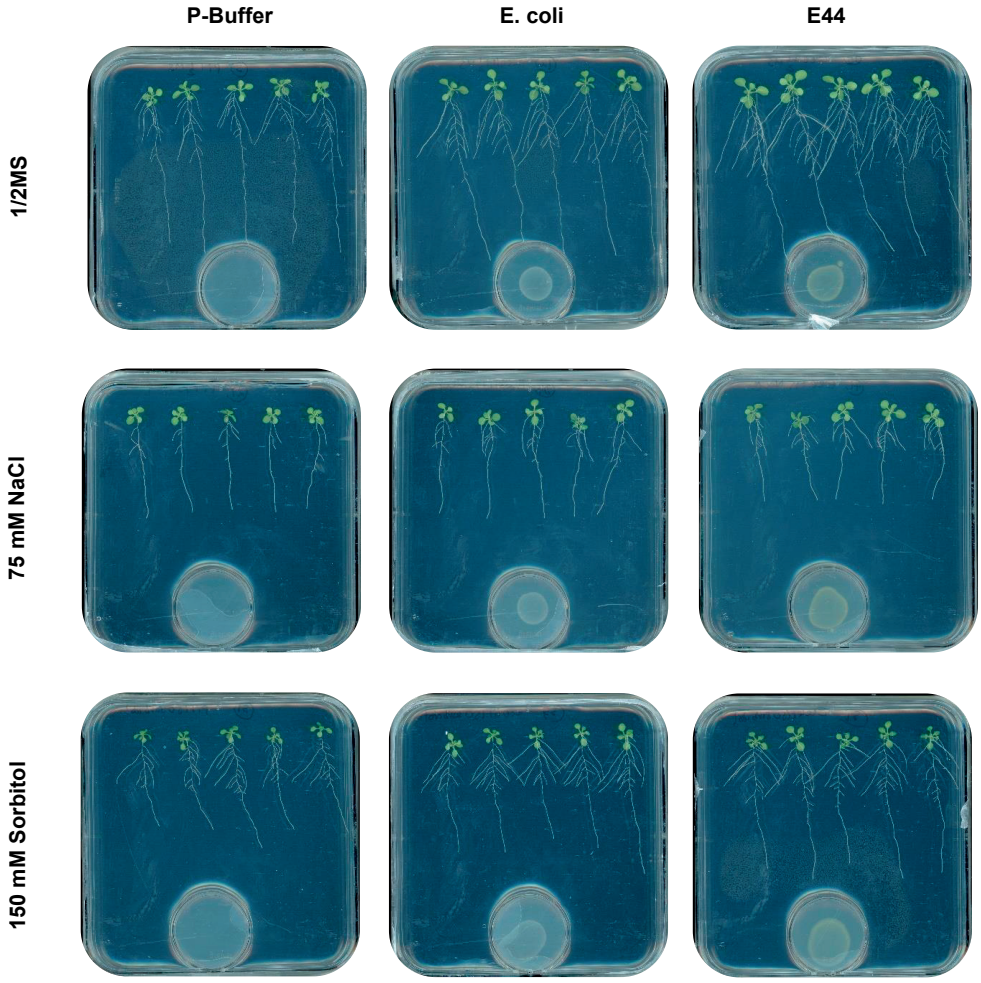
Supplementary Figure 4: Growth phenotype of *Arabidopsis thaliana* Columbia-0 (Col-0) seedlings 13 days after transfer (DAT) to different growth media. Three-day-old seedlings were transferred to ½ MS medium (control), ½ MS supplemented with 75 mM NaCl (salt stress), or ½ MS supplemented with 150 mM sorbitol (osmotic stress) and grown for 13 days. Seedlings were exposed to different volatile treatments in a sealed plate system without direct contact: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* strain DH5α (*E. coli*), or volatiles produced by *Pantoea agglomerans* E44 (E44). Representative images of seedlings are shown.



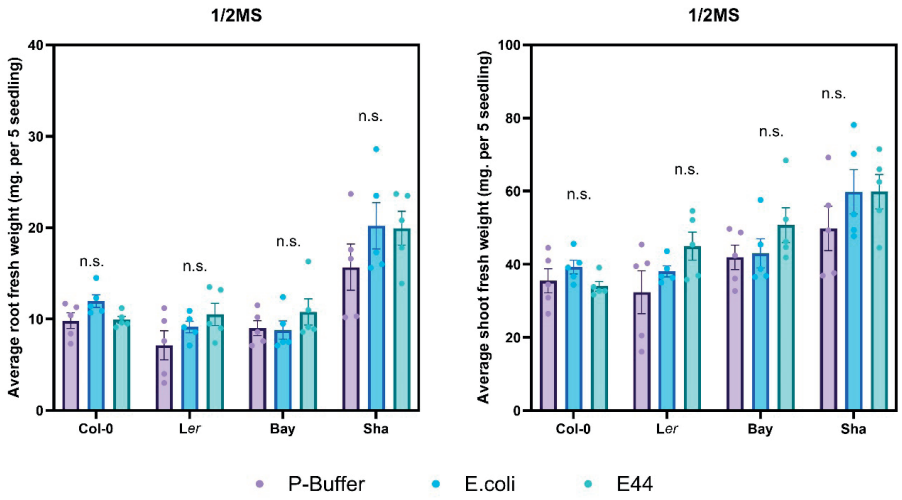
Supplementary Figure 5: Growth phenotype of *Arabidopsis thaliana* Landsberg *erecta* (L*er*) seedlings 13 days after transfer (DAT) to different growth media. Three-day-old seedlings were transferred to ½ MS medium (control), ½ MS supplemented with 75 mM NaCl (salt stress), or ½ MS supplemented with 150 mM sorbitol (osmotic stress) and grown for 13 days. Seedlings were exposed to different volatile treatments in a sealed plate system without direct contact: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* strain DH5α (*E. coli*), or volatiles produced by *Pantoea agglomerans* E44 (E44). Representative images of seedlings are shown.



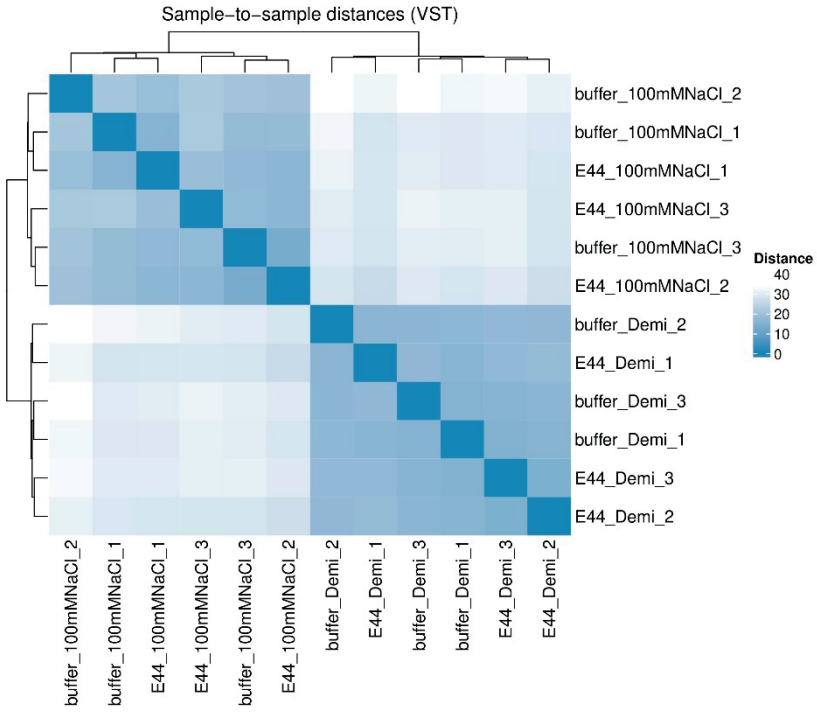
Supplementary Figure 6: Growth phenotype of *Arabidopsis thaliana* Bayreuth (Bay) seedlings 13 days after transfer (DAT) to different growth media. Three-day-old seedlings were transferred to ½ MS medium (control), ½ MS supplemented with 75 mM NaCl (salt stress), or ½ MS supplemented with 150 mM sorbitol (osmotic stress) and grown for 13 days. Seedlings were exposed to different volatile treatments in a sealed plate system without direct contact: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* strain DH5α (*E. coli*), or volatiles produced by *Pantoea agglomerans* E44 (E44). Representative images of seedlings are shown.



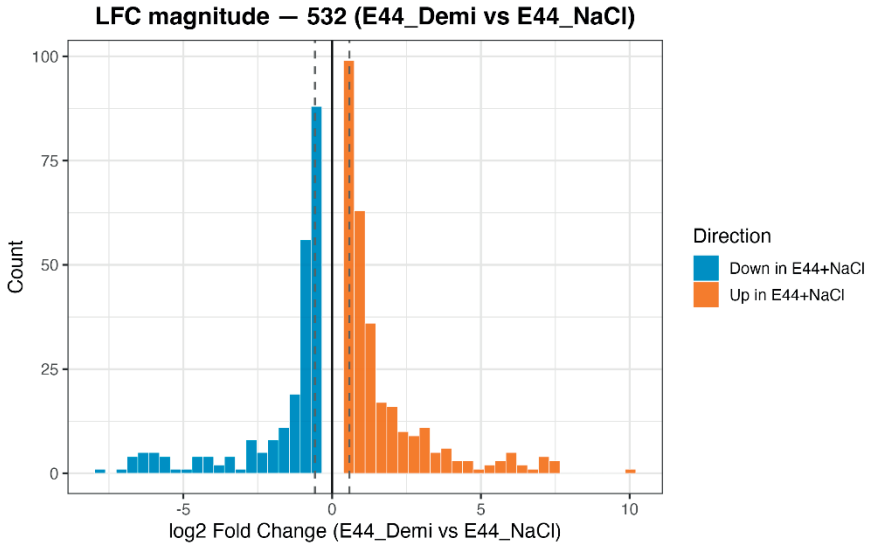
Supplementary Figure 7: Growth phenotype of *Arabidopsis thaliana* Shahdara (Sha) seedlings 13 days after transfer (DAT) to different growth media. Three-day-old seedlings were transferred to ½ MS medium (control), ½ MS supplemented with 75 mM NaCl (salt stress), or ½ MS supplemented with 150 mM sorbitol (osmotic stress) and grown for 13 days. Seedlings were exposed to different volatile treatments in a sealed plate system without direct contact: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* strain DH5α (*E. coli*), or volatiles produced by *Pantoea agglomerans* E44 (E44). Representative images of seedlings are shown.



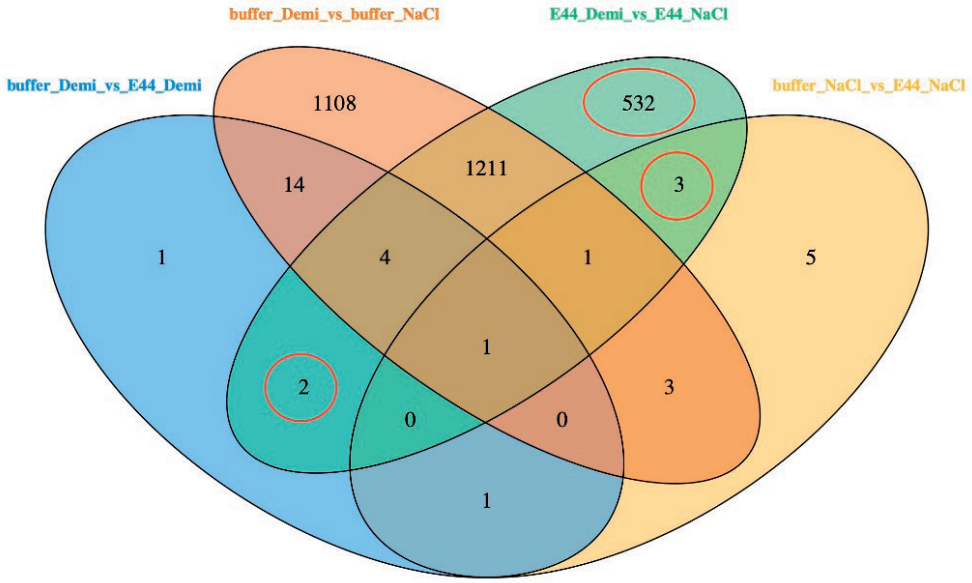
Supplementary Figure 8: Seedling root and shoot fresh weight of four *Arabidopsis thaliana* accessions (Col-0, L*er*, Bay, and Sha) exposed to non-bacterial and bacterial volatiles under non-stress conditions. Seeds were exposed to three volatile treatments: P-buffer (control without bacterial volatiles), volatiles produced by *Escherichia coli* DH5α (E. coli; positive control), and volatiles produced by *Pantoea agglomerans* E44, a bacterial strain isolated from wild cabbage seeds. Seedling root and shoot fresh weight were measured on seedlings grown under non-stress conditions (½ MS medium). Data are presented as mean ± standard error of the mean (SEM). Statistical differences among volatile treatments within each accession were analysed using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and n.s., not significant.



Supplementary Figure 9: Sample-to-sample distance heatmaps of variance-stabilized (VST) RNA-seq gene expression in imbibed *Arabidopsis thaliana* (L*er*) seeds exposed to *Pantoea agglomerans* E44 volatiles or control buffer under salt (100 mM NaCl, imbibed 48 hours) and non-stress (Demi water, imbibed 30 hours) conditions. Heatmaps show pairwise distances between replicates, with closer samples having more similar gene expression profiles. Replicates cluster primarily by stress condition, indicating that salt stress is the dominant factor shaping transcriptional variation, while the effect of E44 volatiles is comparatively minor.



Supplementary Figure 10: Distribution of log_2 fold-change values for genes responding to salt stress in the presence of E44 volatiles. Histogram showing the distribution of log_2 fold-change (LFC) values for 532 genes differentially expressed in response to salt stress in the presence of E44 volatiles (E44__Demi vs E44__NaCl). Genes up-regulated under these conditions are shown in orange, while down-regulated genes are shown in blue. Dashed vertical lines indicate the median LFC for each group. The distribution reveals both positive and negative shifts in gene expression, indicating the presence of up- and down-regulated genes. Most genes exhibit moderate changes in expression, while a subset shows larger fold changes (|LFC| > 2), reflecting stronger transcriptional responses within this gene set.



Supplementary Figure 11: Mapping of the 537 E44-specific salt-responsive genes identified in Fig. 6 onto the Venn analysis shown in Fig. 5. Venn diagram showing the distribution of differentially expressed genes across four pairwise comparisons: buffer_Demi vs buffer_NaCl, E44_Demi vs E44_NaCl, buffer_Demi vs E44_Demi, and buffer_NaCl vs E44_NaCl. The circled regions represent the 537 genes identified as specifically responsive to salt stress only under E44 exposure in the scatter plot analysis (blue points; Fig. 6), comprising 532 genes unique to the E44_Demi vs E44_NaCl comparison and five genes shared with other E44-related contrasts identified in the Venn analysis (Fig. 5).

SUPPLEMENTARY TABLES

All supplementary Tables are available at Supplementary Tables Chapter 3 and contains the following data sheets:

Supplementary Table 1 (Excel file): Differential gene expression analysis of imbibed *Arabidopsis thaliana* seeds under non-stress (Demiwater) and salt stress (100 mM NaCl) conditions with or without exposure to *Pantoea agglomerans* E44 volatiles. Differentially expressed genes (DEGs) were identified using DESeq2 with an adjusted p-value < 0.05 and $|\log_2$ fold change$|$ > 0.58. The table includes gene identifiers, $\log_2$ fold-change values, adjusted p-values, and gene annotations for all pairwise comparisons.

Supplementary Table 2 (Excel file): Classification of differentially expressed genes (DEGs) based on overlap across pairwise comparisons used in the four-way Venn diagram. Genes are grouped according to their presence in the following contrasts: buffer_Demi vs E44_Demi, buffer_NaCl vs E44_NaCl, buffer_Demi vs buffer_NaCl, and E44_Demi vs E44_NaCl. The file includes gene identifiers and annotations corresponding to shared and unique transcriptional responses to E44 volatile exposure and salt stress.

Supplementary Table 3 (Excel file): Gene Ontology (GO) enrichment analysis of up-regulated genes under salt stress in the presence of E44 volatiles. Gene Ontology (GO) enrichment analysis of 300 up-regulated genes identified from the set of 532 genes uniquely differentially expressed under salt stress in the presence of E44 volatiles (E44_Demi vs E44_NaCl).

Supplementary Table 4 (Excel file): Gene Ontology (GO) enrichment analysis of down-regulated genes under salt stress in the presence of E44 volatiles. Gene Ontology (GO) enrichment analysis of 232 down-regulated genes identified from the set of 532 genes uniquely differentially expressed under salt stress in the presence of E44 volatiles (E44_Demi vs E44_NaCl).

Supplementary Table 5 (Excel file): Gene expression data used for scatter plot analysis comparing salt-induced transcriptional responses in the presence and absence of E44 volatiles. The file includes $\log_2$ fold-change ($\log_2$FC) values and adjusted p-values for each gene in the comparisons buffer_Demi vs buffer_NaCl and E44_Demi vs E44_NaCl. Genes are categorized based on their significance in each condition, including salt-responsive genes (SRGs), rescued genes (significant only in buffer), E44-specific salt-responsive genes (significant only in E44), and non-significant genes.

Supplementary Table 6 (Excel file): Gene Ontology (GO) enrichment analysis of rescued genes showing reduced salt-induced transcriptional responses in the presence of E44 volatiles. Rescued genes were defined as genes significantly differentially expressed under salt stress in the P-buffer control but not in the E44 treatment. GO enrichment analysis was performed on genes with available annotations. The table includes enriched GO terms across biological process, cellular component, and molecular function categories, along with gene counts and adjusted p-values.

ACKNOWLEDGEMENTS

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Author contributions

S.S. designed and performed the experiments, analysed the germination and seedling growth data, and wrote the manuscript.

M. C. M. van W. performed the seed germination experiments.

W.P. analysed the RNA-seq data, generated RNA-seq figures, and wrote the manuscript.

L. W. performed the RNA extraction from imbibed seeds.

M.A.S.A., R.K., and L.B. supervised the project, designed the experiments, and edited the manuscript.

Chapter 4

The Core Arabidopsis Seed Microbiome - From Wild to Greenhouse Populations

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ABSTRACT

Seed-associated microbiomes play a critical role in plant fitness, stress tolerance, and early development, particularly during germination. Yet, the influence of the host growth environment and the potential of wild seeds as reservoirs of beneficial microbes remain poorly understood. Here, we characterized the endophytic bacterial communities of seeds from three wild *Arabidopsis thaliana* populations across ecologically distinct locations in Spain and compared them to accessions propagated for three generations under controlled greenhouse conditions using 16S rRNA gene sequencing. Wild seeds harboured population-specific microbiomes with numerous unique and functionally beneficial taxa, whereas greenhouse seeds displayed distinct endophytic communities with fewer unique or population-specific taxa, including non-beneficial and human-associated taxa. Despite these differences, a small conserved core microbiome, dominated by Firmicutes and Bacteroidota species, persisted across all Arabidopsis populations investigated, highlighting host-specific filtering and potential vertical transmission. Cultivable endophytes isolated from wild seeds, included *Peribacillus frigoritolerans*, which significantly enhanced seed germination under osmotic stress via volatile organic compounds, highlighting their functional importance. In summary, seed microbiomes act as a microbial "fingerprint" of the maternal environment. Wild seeds serve as critical reservoirs of stress-resilient, growth-promoting microbes that may be lost during domestication or breeding. These microbes offer opportunities for microbiome-based strategies to enhance crop resilience under environmental stress.

INTRODUCTION

Seed-associated microbiomes play a crucial role in plant growth and health by influencing early developmental stages, including germination and seedling establishment, as well as mediating plant responses to biotic and abiotic stresses (Nelson, 2018; War et al., 2023). Seeds can acquire their microbiome through vertical transmission, in which microbes are passed from the maternal plant to progeny seeds via reproductive tissues, and horizontal transmission, where microbes are recruited from the surrounding environment during seed development (Abdelfattah et al., 2023). Environmental conditions are thought to modulate the success and relative contribution of these two pathways by regulating the maternal plant microbiome and by providing the external microbial pool from which seeds can recruit (Ortúzar et al., 2024; Pérez-Lorente et al., 2024; Prado et al., 2020; Walsh et al., 2021). Factors such as soil properties, local climate, and interactions with surrounding microbial communities act as ecological filters that determine which taxa can establish, persist, and contribute to the seed-associated microbiome (Morales Moreira et al., 2021; Rochefort et al., 2021; Tabassum et al., 2024). Consequently, seed microbiome assembly reflects a combination of vertically inherited and environmentally acquired microbes, structured by the ecological conditions encountered by the host plant throughout its life cycle.

The influence of host growth environment on seed microbiome assembly becomes particularly evident when comparing seeds produced under contrasting conditions (Chartrel et al., 2021). Seeds of wild plants generally harbour rich and diverse microbial communities (Chandel et al., 2022b; Lalzar et al., 2022; Ozkurt et al., 2020). Moreover, wild plant species often exhibit distinct microbial signatures and notable variation in seed microbiome even among populations of the same species (Choi et al., 2022). This variation is likely associated with the diverse environmental conditions experienced by plants across natural habitats (Mészárošová et al., 2024; Wassermann et al., 2019). Such conditions may act as filters (Wani et al., 2022), favouring microbial taxa adapted to fluctuating temperatures, variable moisture and nutrient availability, intense plant–plant interactions, and constant exposure to insects, herbivores, and competing microbes (Hernandez et al., 2021; Saati-Santamaría et al., 2023; Trivedi et al., 2022; Van Nuland et al., 2021). Wild plant seeds contain certain phyla that tend to dominate depending on the species and habitat. For example, wild mustard seeds were primarily colonized by Actinobacteria, Firmicutes, and Gammaproteobacteria (Choi et al., 2022), Moroccan argan seeds contain the Proteobacteria *Rhodoligotrophos* (Sohaib et al., 2024), and native alpine plant seeds encompass Proteobacteria, followed by Actinobacteria and Bacteroidetes (Wassermann

et al., 2019). In contrast, plants grown in cultivated environments, such as domesticated crops, conventional agriculture, or controlled conditions, experience reduced exposure to environmental microbes and harsh conditions (Legein et al., 2022), resulting in seeds with less diverse and different microbial communities compared with wild or field-grown plants (Chen et al., 2024). For example, greenhouse-grown tomato seeds harboured a limited microbial community when compared to the field-grown plants (Bergna et al., 2018). Similarly, seeds of a wild legume (Chandel et al., 2022b) and wild emmer wheat (Ozkurt et al., 2020) harboured more diverse microbiomes than their domesticated species. Interestingly, human-associated taxa such as *Cutibacterium* dominated cultivated cereals seeds, suggesting microbial transfer from humans during seed handling and preparation of nutrient solutions throughout domestication (Abdullaeva et al., 2021). Comparing seed microbiomes across wild and cultivated populations therefore provides a powerful framework to examine how environmental filtering and cultivation practices shape seed microbial community structure.

Despite the strong influence of environmental conditions on seed microbial assembly, many plant species still retain a subset of microbial taxa that consistently associate with seeds, forming a "core seed microbiome". These core members appear to be stably maintained across diverse environments (Eyre et al., 2019; Sulesky-Grieb et al., 2024). For example, although Moroccan argan seeds from different sites exhibited distinct microbial profiles, taxa such as Pseudomonas, Bacillus, and Aspergillus were detected across multiple locations (Sohaib et al., 2024). In soybean, bacterial genera, including *Pseudomonas*, *Pantoea*, *Bacillus*, and *Sphingomonas*, were shared between wild and domesticated populations, forming a conserved core (Chandel et al., 2022b). At a broader scale, a global seed core identified across 50 plant species consisted mainly of Proteobacteria, particularly *Pantoea* and *Pseudomonas*, with additional contributions from Actinobacteriota and Firmicutes (Simonin et al., 2022). Likewise, Johnston-Monje et al. (2021) found that *Pantoea* and *Enterobacter* were universally present across seeds of 17 species. However, despite increasing evidence for conserved seed microbiome, the extent to which seed microbiomes are maintained across contrasting growth environments and multiple generations remains poorly understood.

Characterizing the seed microbiome has practical relevance beyond ecological description. Wild seeds often harbour greater microbial diversity and unique beneficial lineages, providing a reservoir of traits that improve germination, stress tolerance, and disease resistance (Liang et al., 2021; Roodi et al., 2020). For example, *Rhodococcus corynebacterioides* B2 and *Staphylococcus haemolyticus* D4 from wild mustard seeds enhanced *Arabidopsis thaliana* shoot and root growth (Choi et al., 2022); Volatile organic compounds (VOCs) emitted from *Pantoea*

agglomerans E44 isolated from wild cabbage seeds stimulated germination (Tyc et al., 2020); and *Methylobacterium* strains from wild Brassica seeds promoted oilseed rape growth and suppressed the blackleg pathogen (Roodi et al., 2020). These observations highlight wild species as an important source of beneficial microbes that may have been lost during cultivation and nutrient-rich environments. However, the functional roles of cultivable seed endophytes from wild species, particularly their ability to influence germination, remain poorly understood.

To address these knowledge gaps, this chapter integrates amplicon-based microbial community profiling with culture-dependent isolation and germination assays under stress. We hypothesized that wild *Arabidopsis thaliana* populations would harbour more diverse and population-specific seed microbiomes than greenhouse-grown populations, while a small subset of bacterial taxa would be consistently maintained across populations and growth environments. We further hypothesized that cultivable seed endophytes isolated from wild populations would have the potential to influence Arabidopsis germination through volatile-mediated interactions. To test these hypotheses, we characterized the seed-associated bacterial microbiome of *Arabidopsis thaliana* from wild populations, each representing distinct ecological contexts, and compared them with greenhouse-grown seeds cultivated in four different substrates (as described in Chapter 2). Using 16S rRNA amplicon sequencing, we aimed to (i) determine how host growth conditions influence seed microbiome diversity and composition, and (ii) identify core bacterial taxa shared across populations. In parallel, we isolated cultivable seed endophytes from wild populations and evaluated the potential effects of VOCs produced by two selected isolates on Arabidopsis germination under osmotic stress, a key environmental challenge in Mediterranean ecosystems (Páscoa et al., 2017). Together, these approaches provide an integrated framework for assessing how environmental conditions, core microbiome persistence, and seed-borne microbial functions shape plant performance, offering insights relevant to both natural ecosystems and controlled plant production systems.

MATERIALS AND METHODS

Wild population seeds

In May 2022, seeds from three distinct wild *Arabidopsis thaliana* populations, Marjaliza (MAR), Moral de Calatrava (MDC) and Poblet (POB), were collected from separate locations in Spain (Fig. 1a). These populations have been previously described at the ecological and genomic diversity levels (de la Mata et al., 2024) and provided 16, 15 and 12 individual lines, respectively (Fig. 1a; Supplementary Table 1). Following harvest, seeds were transported to the Laboratory of Plant

Physiology at Wageningen University & Research, the Netherlands. Upon arrival, seeds were initially stored in a controlled environment at 20°C and 30% relative humidity. Subsequently, they were transferred to an after-ripening cabinet maintained at 20°C with 55% relative humidity to ensure appropriate seed after-ripening conditions. Seeds were stored in the after-ripening cabinet for 14 months prior to conducting the experiment.

Soil samples

To characterize the soil microbial communities associated with *Arabidopsis thaliana* in natural environments, soil samples were collected from the same populations and sampling sites as the seed collections. A total of 18 bulk soil samples were collected and processed as independent samples, including 8 samples from the MAR population and 10 samples from the MDC population (Supplementary Table 1). No soil samples were collected from the POB population. Bulk soil samples were collected at a mean distance of 5.3 ± 1.4 cm from the target plants (mean ± SE; range: 0–20 cm; Supplementary Table 1) and transferred into sterile 50 mL Greiner tubes. The largest distances (20 cm) occurred only in a few cases where plants were growing among rock crevices and soil could not be sampled directly adjacent to the target plant. Samples were immediately placed on ice in the field to maintain microbial integrity during transport. All samples were transported to the laboratory on ice and stored temporarily at 4°C. Subsequently, the soil samples were shipped under cold conditions to the Laboratory of Plant Physiology at Wageningen University & Research, the Netherlands. Upon arrival, soil samples were stored at -80°C until microbial DNA extraction.

Seed procedure before microbial DNA extraction and bacterial isolation

Due to the limited number of seeds obtained from each individual line of the wild populations, seeds from all lines within a population were pooled and treated as a single population sample. Prior to microbial DNA extraction, seeds were surface-sterilized using a wet sterilization procedure to remove surface-associated microbes and ensure that only endophytic bacterial communities were analysed. All steps were conducted under a laminar flow cabinet to maintain sterility. For each sample, 50 mg of seeds were placed in a 2 mL Eppendorf tube and incubated with 1 mL of 0.1% (v/v) sodium hypochlorite mixed with 0.1% (v/v) Tween 80. The tube was gently tapped to mix, ensuring minimal foaming, followed by a 1 minute incubation. After centrifugation, the supernatant was removed using sterile filter-tips. The seeds were then rinsed five times with 1 mL of sterile demineralized water per wash. A new sterile pipette tip was used for each wash to prevent cross-contamination. To confirm the effectiveness of surface sterilization, 100 µL of the final rinse water was plated on 1/10th tryptic

soy broth agar (TSBA) (5.0 g/L NaCl; 1.0 g/L KH_2PO_4; 3 g/L Oxoid Tryptic Soy Broth; and 20 g/L BACTO agar, pH 6.5) and incubated at room temperature for seven days. The absence of bacterial or fungal growth confirmed successful surface sterilization of the seeds.

Microbial DNA extraction from seeds

Approximately 50 mg of wet surface-sterilized seeds were transferred to a sterile mortar, and 1 mL of 10 mM phosphate buffer (P-buffer) (pH 6.5) was added. Seeds were manually ground using a sterile pestle until a fine powder was obtained. An additional 4 mL of P-buffer was then added to the mortar and briefly mixed. The mixture of ground seeds and P-buffer was transferred to a 50 mL sterile Greiner tube and incubated at 20°C with shaking at 180 rpm for 45 minutes to obtain the undiluted seed suspension (10^0). For each population, 250 µL of this suspension was used per replicate for microbial DNA extraction. The MAR and MDC populations were processed with 5 technical replicates each, and the POB population was processed with 4 technical replicates. The same undiluted seed suspensions were later used for bacterial isolation. Microbial DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, NL) following the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

Microbial DNA extraction from soils

For each MAR and MDC population, 250 mg of soil was used per sample, with three technical replicates performed for each population. No soil samples were collected from the POB population. Microbial DNA was extracted using the DNeasy PowerSoil Pro Kit (QIAGEN, NL) following the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA).

16S rRNA library preparation and sequencing for microbial communities

16S rRNA gene amplicon libraries were prepared and sequenced by Novogene (UK) to analyze the microbial communities of seed and soil samples. For seed microbial communities, the V5–V7 region of the 16S rRNA gene was amplified using the primers 799F (AACMGGATTAGATACCCKG) and 1193R (ACGTCATCCCCACCTTCC). For soil microbial communities, the V3–V4 region was amplified using the primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC). To minimize non-target amplification of mitochondrial and plastid DNA, peptide nucleic acid (PNA) clamps were used, including mPNA (GGCAAGTGTTCTTCGGA) and pPNA (GGCTCAACCCTGGACAG). Samples were sequenced on a NovaSeq 6000 (Illumina) platform, generating approximately 100,000 raw sequence reads

per sample for seed samples and approximately 30,000 raw sequence reads per sample for soil samples, ensuring sufficient coverage for downstream microbial community analysis.

Amplicon sequence processing and analysis

Raw 16S rRNA gene sequencing data were processed using NG-Tax 2.0 (v2.2.10) (Poncheewin et al., 2019), with read lengths for both forward and reverse reads set to 140 bp (-for_read_len and -rev_read_len) and a minimum detection threshold (-minPerT) of 0.01. Amplicon sequence variants (ASVs) were identified on a per-sample basis, and taxonomic classification was performed using the SILVA 138.1 reference database for the 16S rRNA gene (Quast et al., 2013). The resulting NG-Tax output was imported into RStudio (v4.4.1), where a phyloseq object was constructed by integrating the ASV table, phylogenetic tree, and sample metadata using the phyloseq package (v1.48.0) (McMurdie & Holmes, 2013). ASVs assigned to chloroplast (class Cyanobacteriia) and mitochondria (order Rickettsiales) were excluded from all samples, as these correspond to plant/host organellar sequences rather than bacterial taxa. In addition, an ASV was retained for a given population only if detected in at least two replicates of that population; no relative-abundance cut-off was applied.

Alpha and beta diversity analyses were performed using the phyloseq and vegan (v2.7-2) packages in RStudio (v4.4.1). Alpha diversity was quantified using the Observed Richness Index obtained via the estimate_richness function. To evaluate differences in alpha diversity among sample groups, an ANOVA model (aov) was fitted using Observed richness values, followed by Tukey's Honest Significant Difference (HSD) post hoc tests. Pairwise statistical results from Tukey HSD were exported for reporting. To visualize statistically significant groupings, compact letter displays were generated using the multcompView (v 0.1-10) package and overlaid onto boxplots created with ggplot2. Beta diversity was assessed using Bray-Curtis dissimilarity, calculated from ASV abundance tables using vegdist. Community structure differences were visualized by non-metric multidimensional scaling (NMDS) using the metaMDS function. Metadata were merged with NMDS coordinates for visualization, and samples were coloured by experimental condition and shaped by population origin. The statistical significance of community separation was evaluated using permutational multivariate analysis of variance (PERMANOVA) via the adonis2 function with 999 permutations. Unique and shared taxa among conditions were assessed using presence-absence data and visualized with Venn diagrams generated using the Venn (v1.12) and VennDetail (v1.20.0) packages. Taxonomic labels were aggregated across hierarchical levels prior to comparison. All data manipulation, including factor handling, filtering, and metadata preparation, was performed

using the tidyverse suite (v2.0.0) (Wickham et al., 2019). Visualization of bar plots, boxplots (with significance letters), scatter plots, and NMDS ordinations was performed using ggplot2.

Comparison of *Arabidopsis thaliana* seed microbiomes between wild and greenhouse populations

To assess the influence of host growing environment on seed-associated bacterial communities, we compared the 16S rRNA gene-based taxonomic profiles of wild and greenhouse-grown *Arabidopsis thaliana* seeds. Wild seeds correspond to the three natural Mediterranean populations described above. For greenhouse populations, seeds were obtained from four accessions, Columbia-0 (Col-0), Landsberg *erecta* (L*er*), Bayreth (Bay), and Shahdara (Sha), propagated for three consecutive generations (G3) under controlled greenhouse conditions. Plants were cultivated on four substrate types: rockwool, commercial potting substrate, gamma-sterilized field soil, and non-sterile field soil (see Chapter 2 for full experimental details). This design enabled direct comparisons between microbiomes of seeds produced in natural habitats and those produced under controlled greenhouse propagation across multiple substrates.

Alpha diversity was quantified using the Observed Diversity Index. Statistical differences in alpha diversity among groups were assessed using one-way ANOVA followed by Tukey's post hoc test, with significance defined as $P < 0.05$. Beta diversity of seed bacterial communities was visualized using non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity. Differences in community composition between wild and greenhouse samples, as well as among substrate types, were evaluated using permutational multivariate analysis of variance (PERMANOVA). Unique and shared taxa among populations were assessed using presence-absence data and visualized with Venn diagrams generated using the Venn (v1.12) and VennDetail (v1.20.0) packages.

Isolation, genomic DNA extraction, and 16S rRNA gene amplification of endophytic bacteria from wild Arabidopsis seeds

All bacterial isolation, DNA extraction, and PCR amplification procedures of seed endophytic bacteria were carried out at the Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands, following standard aseptic and molecular biology protocols.

Isolation of seed bacterial endophytes

Seed bacterial endophytes were isolated from wild *Arabidopsis thaliana* seeds using serial dilutions of the seed suspension (see sections "Seed procedure before

microbial DNA extraction and bacterial isolation" and "Microbial DNA extraction from seeds"). The undiluted seed suspension (10^0) was plated directly, while 10^{-1} and 10^{-2} dilutions were prepared by sequentially transferring 100 µL of the previous dilution into 900 µL of 10 mM P-buffer (pH 6.5). For each dilution, 100 µL was plated in triplicate on different media: 1/10th TSBA (pH 6.5), Reasoner's 2A agar (R2A;18.12 g R2A agar, 1 L demineralized water), half-strength Potato Dextrose Agar (0.5×PDA;19.5 g PDA, 7.5 g Bacto agar, and 1 L demineralized water, pH 6.5), and Luria-Bertani media (LB; 25 g Tryptone, 12.5 g Yeast extract, 25 g NaCl, 10 g Micro agar, and 1 L demineralized water, pH 7.0). Plates were incubated at 20°C for seven days. After incubation, plates were examined for colony growth, and two colonies with similar morphology were selected from each plate. Each selected colony was streaked onto fresh 1/10th TSBA (pH 6.5) and incubated at 20°C for five days. Single colonies from the first streak plates were then re-streaked onto new 1/10th TSBA (pH 6.5) plates to obtain purified isolates, which were incubated under the same conditions. For long-term storage, a single colony from each purified isolate was inoculated into 3 ml of liquid 1/10th TSB and incubated overnight at 28 °C with shaking at 190 rpm. The following day, 900 µL of the bacterial culture was mixed with 900 µL of sterile 40% (v/v) glycerol in sterile 2 mL cryogenic tubes (Thermo Fisher Scientific Inc., USA) to prepare glycerol stocks, which were stored at -80 °C. Purified bacterial isolates recovered from these glycerol stocks were subsequently used for taxonomic identification based on 16S rRNA gene sequencing.

Genomic DNA extraction

Genomic DNA was extracted from purified bacterial isolates recovered from glycerol stocks. Colonies were streaked onto 1/10th TSBA (pH 6.5) and incubated at 28°C for 48 hours to obtain fresh single colonies. Single bacterial colonies were then cultured in 3 mL of liquid 1/10th TSB (pH 6.5) at 28 °C with shaking at 190 rpm for 24-48 hours. One milliliter of each bacterial culture was transferred to a 2 mL microcentrifuge tube and centrifuged at 12,000 × g for 10 minutes at room temperature. The supernatant was discarded, and the resulting bacterial pellet was used directly for genomic DNA extraction using the QIAamp DNA Micro Kit (QIAGEN, NL) following the manufacturer's protocol for bacterial cells. DNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., USA). Extracted DNA samples were stored at -20 °C until PCR analysis.

PCR Amplification of the 16S rRNA Gene

PCR amplification of the 16S rRNA gene was performed using universal bacterial primers 27F (5′-AGA GTTTGATCMTGGCTCAG -3′) and 1492R (5′-GRTACCTTG TTA CGA CTT -3'), targeting approximately 1465 bp of the 16S rRNA gene. Each

50 µL PCR reaction contained 25 µL GoTaq G2 Green Master Mixs (Promega, USA), 20 µL nuclease-free water, 2 µL of each primer (10 µM), and 1 µL of genomic DNA. PCR was performed in a Bio-Rad C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., NL) with the following thermal cycling conditions: initial cycle 95 °C for 2 minutes, 30 cycles of 94 °C for 30 seconds, 55 °C for 1 minute, 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. Reactions were then held at 12 °C for 10 minutes prior to storage or downstream analysis.

Agarose gel electrophoresis, PCR product purification, and sequencing

PCR products were verified by electrophoresis on a 1% (w/v) agarose gel in 0.5x TBE buffer and run at 100 V for 60 minutes. A 5 µL aliquot of each PCR reaction and 1 µL of GeneRule DNA Ladder Mix (100–10,000 bp; Thermo Fisher Scientific Inc., USA) were loaded onto the gel to assess the presence and size of amplified fragments. PCR products showing a single band of the expected size (~1.5 kb) were purified using the QIAGEN PCR purification kit (QIAGEN, NL) according to the manufacturer's instructions and subsequently quantified with a NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., USA). Purified PCR products were diluted to a final concentration of 50 ng/µl, combined with either primer 27F or 1492R, and sent for Sanger Sequencing at Macrogen Europe (NL). Resulting chromatograms were manually inspected, trimmed, and assembled using BioEdit software (Hall, 1999). Consensus sequences were compared against the NCBI nucleotide database using BLASTn algorithm (Federhen, 2012) to determine the closest taxonomic matches.

Seed germination assay in response to bacterial volatiles

To evaluate the effect of volatiles emitted by seed endophytic bacteria on *Arabidopsis thaliana* seed germination, a seed germination assay was performed under controlled laboratory conditions. Seeds were exposed to bacterial volatile organic compounds (VOCs) using a sealed plate system in which bacterial cultures and seeds were physically separated, allowing exposure to VOCs without direct seed-bacterial contact. Germination was assessed under non-stress, using demineralized water, and osmotic stress, induced by 500 mM D-mannitol, in the presence or absence of bacterial volatiles.

Bacterial strains and preparation

The endophytic strain *Peribacillus frigoritolerans*, the only bacterium shared between the MAR and MDC wild populations in our isolate collection (Table 3), was used in the assay using two independently isolated representatives (strains MDC39 and MAR22; Table 3; Supplementary Fig. 3 and 4). An additional bacterial strain, *Pantoea agglomerans* E44, isolated from wild cabbage seeds as described

in Tyc et al. (2020), was included as a positive control. Bacterial strains were recovered from glycerol stocks, streaked onto 1/10th TSBA (pH 6.5), and incubated at 28 °C for 48 hours. Single colonies were inoculated into 3 mL of liquid 1/10th TSB (pH 6.5) and incubated overnight at 28 °C with shaking at 190 rpm. Cells from the overnight culture were harvested by centrifugation at 4,000 rpm for 5 min, and the resulting pellets were washed twice and resuspended in 10 mM phosphate buffer (pH 6.5). Final bacterial suspensions were adjusted to an optical density of OD_{600} = 0.005 in 10 mM phosphate buffer (pH 6.5). Sterile phosphate buffer was used as a negative control, representing the absence of bacterial volatiles. Then, 50 µL of each bacterial suspension or buffer control was plated onto the lid of a 60 × 15 mm round Petri dish (Greiner Bio-One B.V., NL) containing 10 mL of 1/10th TSBA (pH 6.5). Plates were incubated at 20 °C for 48 hours to allow bacterial growth. At this stage, the bacterial plates were not yet exposed to seeds and were transferred to the germination plates in the subsequent assay step.

Seed sterilization and germination setup

Prior to sowing, *Arabidopsis thaliana* (accession Landsberg *erecta*; L*er*) seeds were surface-sterilized using chlorine gas. Opened Eppendorf tubes containing seeds were placed inside a closed desiccator with a glass beaker containing 100 mL of bleach (sodium hypochlorite) and 3 mL of 37% hydrochloric acid to generate chlorine gas. Seeds were exposed to the gas for 5 h. Sterilized seeds were then sown onto two layers of blue germination paper placed in 120 × 120 × 17 mm square plates (Greiner Bio-One B.V., NL). The germination paper was pre-soaked with either demineralized water (non-stress condition) or 500 mM D-mannitol (osmotic stress). To test the effects of different concentrations of bacterial volatiles, two arrangements of bacterial/control plates were used. In the single-plate setup (Supplementary Fig. 1a), one bacterial or control plate was placed in the top-left corner, and the germination paper was soaked with 15 mL of solution. In the double-plate setup (Supplementary Fig. 1b), two bacterial or control plates were placed in the top-left and bottom-right corners, and the germination paper was soaked with 10 mL of solution. For the single-plate setup, three replicates of approximately 50 seeds each were used, whereas for the double-plate setup, two replicates of approximately 50 seeds each were used. In both setups, bacterial strains were grown separately, and seeds were exposed exclusively to their volatiles without direct bacterial-seed contact. All plates were sealed with parafilm to prevent volatile loss, randomized, and incubated at 22 °C under continuous light for 7 days. Images were captured twice daily, in the morning and afternoon, and analyzed using the GERMINATOR software package (Joosen et al., 2010). Germination metrics, including the percentage of maximum germination (Gmax) and the time to reach 50% of maximum germination (T50), were calculated.

RESULTS

Distinct seed microbiome composition across wild *Arabidopsis thaliana* populations

To characterize the structure and composition of seed endophytic bacterial communities in wild *Arabidopsis thaliana* across different environments, we performed 16S rRNA gene amplicon sequencing on seeds collected from three distinct locations in Spain, with the sampling sites shown in Fig. 1a and Supplementary Table 1. Taxonomic profiles were generated at the genus level. The top 10 most abundant genera across all samples were visualized in a stacked bar plot (Fig. 1b). Overall, the seed endophytic communities differed among the three environments. In the MAR population, *Pseudomonas* was the most abundant genus, followed by *Buchnera*. In contrast, the MDC population was dominated by *Buchnera* and taxa belonging to the family *Muribaculaceae*. The POB population displayed a distinct profile, characterized by high abundances of *Muribaculaceae*, *Faecalibaculum*, and members of the *Lachnospiraceae*. Remarkably, *Pseudomonas* were detected exclusively in the MAR population, indicating site-specific enrichment. *Buchnera* was present in both MAR and MDC populations but was absent from the POB population (Supplementary Table 2 and 3).

Alpha diversity, reflecting the number of unique taxa per sample, did not differ significantly among the three populations (Observed Diversity Index; Fig. 1c), indicating that seeds were similarly rich in bacterial taxa across sites. In contrast, beta diversity of seed endophytic bacterial communities, assessed using Bray–Curtis dissimilarity and visualized with non-metric multidimensional scaling (NMDS; Fig. 1d), revealed that seed endophytic bacterial community composition differed significantly among the three populations. The NMDS ordination provided a reliable representation of community dissimilarities (stress = 0.022), indicating that the observed clustering reliably reflects differences in microbial composition. These compositional differences were statistically supported by PERMANOVA (R^2 = 0.361, F = 3.11, p = 0.005, permutations = 999), indicating that differences among populations accounting for approximately 36% of the observed variation in seed endophytic microbiome structure. The remaining variation likely reflects natural variability among samples as well as other environmental and host-related factors that were not assessed in this study. The remaining variation likely reflects within-population heterogeneity and other unmeasured factors. Pairwise comparisons revealed significant differences between MAR and POB (R^2 = 0.292, p = 0.025), MAR and MDC (R^2 = 0.332, p = 0.008), and POB and MDC (R^2 = 0.265, p = 0.047). The strongest difference was observed between MAR and MDC populations (R^2 = 0.332), while the smallest difference occurred between POB and MDC populations (R^2 = 0.265). Together, these results indicate

that although the number of bacterial taxa per seed sample was similar across populations, each population harboured a distinct seed microbiome composition shaped by local environmental conditions.

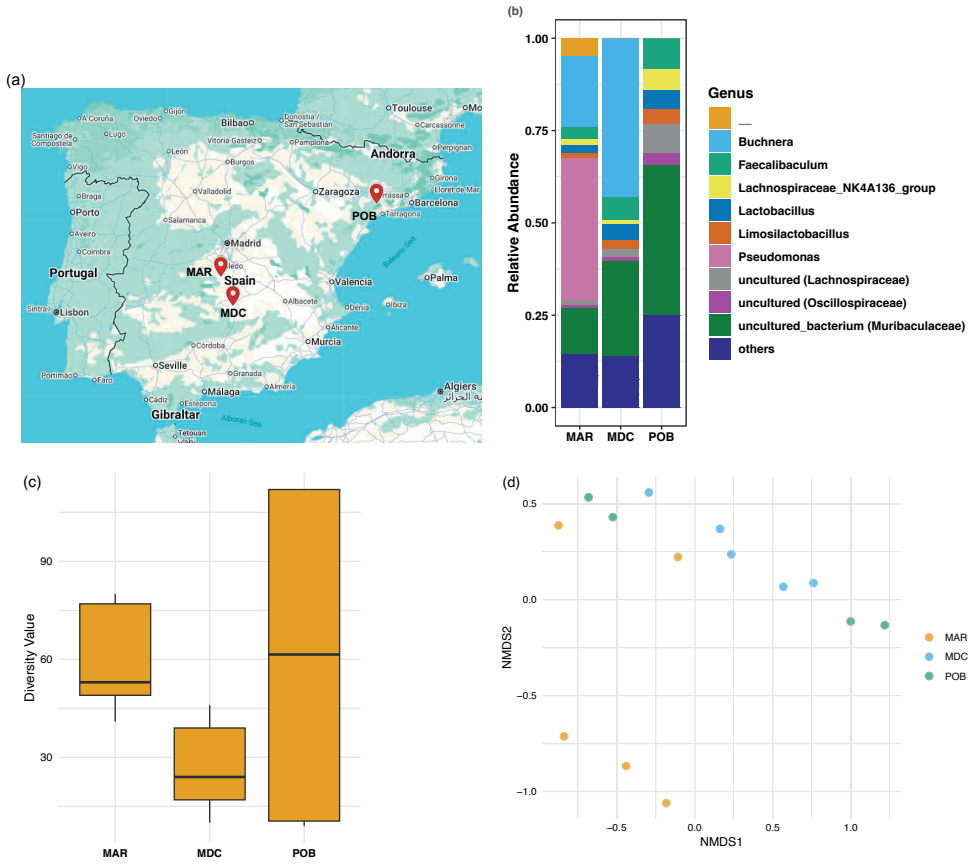


Figure 1: Structure and composition of seed endophytic bacterial communities in three wild *Arabidopsis thaliana* populations collected in Spain in 2022. (a) Map of Spain showing the three sampling locations, Marjaliza (MAR), Moral de Calatrava (MDC) and Poblet (POB). (b) Relative abundance of the ten most abundant bacterial genera (family indicated in parentheses) in seeds from three wild populations. Genera outside the top ten are grouped as "Other" for clarity. Taxa labelled as "uncultured" were identified only by sequencing and cannot be assigned to formal genus, but are reported at higher taxonomic ranks (e.g., family). Taxa labelled as "___" could not be classified beyond the order level. See Supplementary Tables S2 for detailed taxonomic assignments. (c) Alpha diversity measured using the Observed Diversity Index. Differences among the three seed populations were assessed using one-way ANOVA followed by Tukey's post hoc test. n.s. indicates no significant difference among MAR, MDC, and POB seed samples ($P \geq 0.05$). (d) Beta diversity of seed endophytic bacterial communities visualized by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities. NMDS was performed using three dimensions ($k = 3$), and the final ordination showed a low stress value (0.022), indicating a reliable representation of community dissimilarities. Each point represents an individual seed sample, and clustering reflects differences in bacterial community composition among populations. Community differences were statistically supported by PERMANOVA ($R^2 = 0.361$, $F = 3.11$, $p = 0.005$, permutations = 999).

Conserved core bacterial endophytes across wild populations

To identify the core bacterial taxa of wild *Arabidopsis thaliana* seeds, we examined the overlap of genera among the three wild populations (MAR, MDC, and POB). A Venn diagram revealed that 18 genera (29.5% of 61 total genera) were shared across all three populations, representing the core seed microbiome (Fig. 2, Table 1). These conserved core genera were predominantly associated with the phylum Firmicutes (11 genera), including *Faecalibaculum*, *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus*, *Lachnospiraceae_NK4A136_group*, *A2*, *Lachnospiraceae_UCG-006*, *Blautia*, and *Carnobacterium*, as well as several taxa classified only as uncultured bacteria. The core also contained five genera from the phylum Bacteroidota, including *Muribaculum*, *Alloprevotella*, *Prevotellaceae_UCG-001*, *Alistipes*, and uncultured representatives. Additionally, one core genus was affiliated with Deferribacterota (*Mucispirillum*) and one with Campylobacterota (*Helicobacter*) (Table 1). These taxa were consistently present regardless of environmental differences among sites, suggesting that they are stably associated with wild *Arabidopsis thaliana* seeds.

Each population also harboured its own set of unique genera. MAR and POB each contained 15 unique genera (24.5% of all detected genera), whereas MDC contained only two unique genera (3.3%). The unique genera in the MAR population belonged to the phyla Proteobacteria (e.g., *Pseudomonas*, *Roseomonas*, and additional unclassified taxa within *Gammaproteobacteria*), Actinobacteriota (*Cellulomonas*, *Clavibacter*, *Pseudonocardia*, *Conexibacter*, *Solirubrobacter*, and *Microbacterium*), and Acidobacteriota (two unclassified taxa). In the POB population, most unique genera were affiliated with Firmicutes, including *Lachnospiraceae_UCG-001*, *Roseburia*, *Marvinbryantia*, *Lachnoclostridium*, *Lachnospiraceae_FCS020_group*, *Eubacterium_xylanophilum_group*, *UCG-009*, and several additional unclassified taxa. A smaller number of unique genera belonged to Bacteroidota (*Prevotella_9*) and Desulfobacterota (*Desulfovibrio*). In contrast, the MDC population contained only two unique genera, both within Proteobacteria: *Rickettsiella* and *Pantoea*. The complete list of population-specific genera is provided in Supplementary Table 3. Together, these results indicate that while local environmental conditions shape portions of the seed microbiome, certain bacterial groups are consistently present across all populations, highlighting a conserved core seed microbiome in wild *Arabidopsis thaliana.*

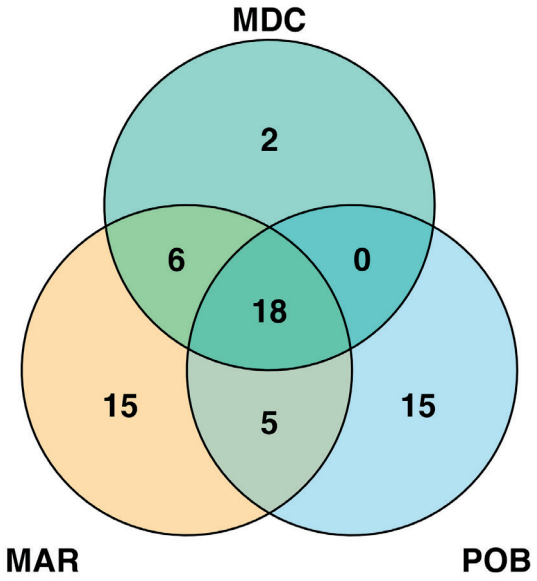


Figure 2: Shared and unique seed endophytic bacterial genera across three wild *Arabidopsis thaliana* populations (MDC, MAR, and POB). Venn diagrams illustrate the overlap and population-specific seed endophytic bacterial genera among wild populations, highlighting the conserved core microbiota present across all populations.

Table 1: Core seed endophytic bacterial genera shared across all wild Arabidopsis populations. Endophytic bacterial genera consistently detected in seeds of all three wild *Arabidopsis thaliana* populations: MDC, MAR and POB. Taxa were identified by 16S rRNA gene amplicon sequencing and are reported at five taxonomic ranks: phylum, class, order, family, and genus.

No.	Phylum	Class	Order	Family	Genus
1	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*uncultured_bacterium*
2	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
3	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
4	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
5	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*
6	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
7	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
8	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
9	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
10	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured_bacterium*
11	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_NK4A136_group*
12	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*
13	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*
14	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*
15	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*uncultured_bacterium*
16	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
17	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
18	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*

Distinct microbial communities in seeds and soil with limited soil contribution

To investigate the relationship between seed-associated and bulk soil bacterial communities, we compared the seed microbiomes of the two wild *Arabidopsis thaliana* populations (MDC and MAR) with bulk soil collected from the same sites. The relative abundance profiles revealed clear differences between the microbial communities of wild Arabidopsis seeds and their corresponding soil environments for both MAR and MDC populations (Fig. 3a). Seed communities were dominated by a limited number of genera that appeared at consistently high relative abundances. In contrast, soil microbiomes displayed a broader number of taxa, with many genera present at low to moderate abundances. Alpha and beta diversity analyses supported these findings. Soil bacterial communities were significantly more diverse than seed communities (Observed Diversity Index; Fig. 3b). Beta diversity of seed and soil bacterial communities, assessed using Bray-Curtis dissimilarity and visualized with NMDS (Fig. 3c), revealed a clear and significant separation between seeds and bulk soil. The NMDS ordination provided a highly stable representation of community dissimilarities (stress ≈ 0.0001), indicating that the observed clustering reliably reflects differences in microbial composition. These compositional differences were statistically supported by PERMANOVA (R^2 = 0.078, F = 1.702, p = 0.037, permutations = 999), indicating that seed bacterial communities are distinct from those in the surrounding soil.

The Venn diagram comparing seed and bulk soil communities revealed that only seeds from MAR population shared a small core set of nine bacterial genera with their surrounding soil. The shared taxa represented just 3.6% of the 251 genera detected across the combined seed and soil microbiomes (Fig 3d), including *Pseudomonas*, *Lactobacillus*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Cellulomonas*, *Pseudonocardia*, *Conexibacter*, *Solirubrobacter*, *Roseomonas* and *Microbacterium* (Supplementary Table 4). In addition to the shared genera, MAR seeds harboured 35 genera not detected in MAR soil, including *Clavibacter, Odoribacter, Bacteroides,* and *Parasutterella*, while MDC seeds contained 26 genera not detected in MDC soil, including *Lactobacillus, Rickettsiella,* and *Pantoea* (Supplementary Tables 4-5). Among these seed-exclusive genera, many taxa were shared across both populations, including *Mucispirillum*, *Faecalibaculum*, *Lachnospiraceae_UCG-001*, *Ligilactobacillus*, *Carnobacterium*, *Blautia*, *Muribaculum*, *Prevotellaceae_UCG-001*, *Alistipes*, *Helicobacter*, *Buchnera*, and *Escherichia–Shigella* (Supplementary Tables 4-5), suggesting that certain bacterial taxa are preferentially enriched and recruited in seeds regardless of the surrounding soil community. Together, these findings demonstrate that only a very small subset of soil bacteria colonize inside the seeds. Instead, wild *Arabidopsis thaliana* seeds harbour a distinct and selectively enriched microbiome, indicating strong host filtering during seed microbiome assembly.

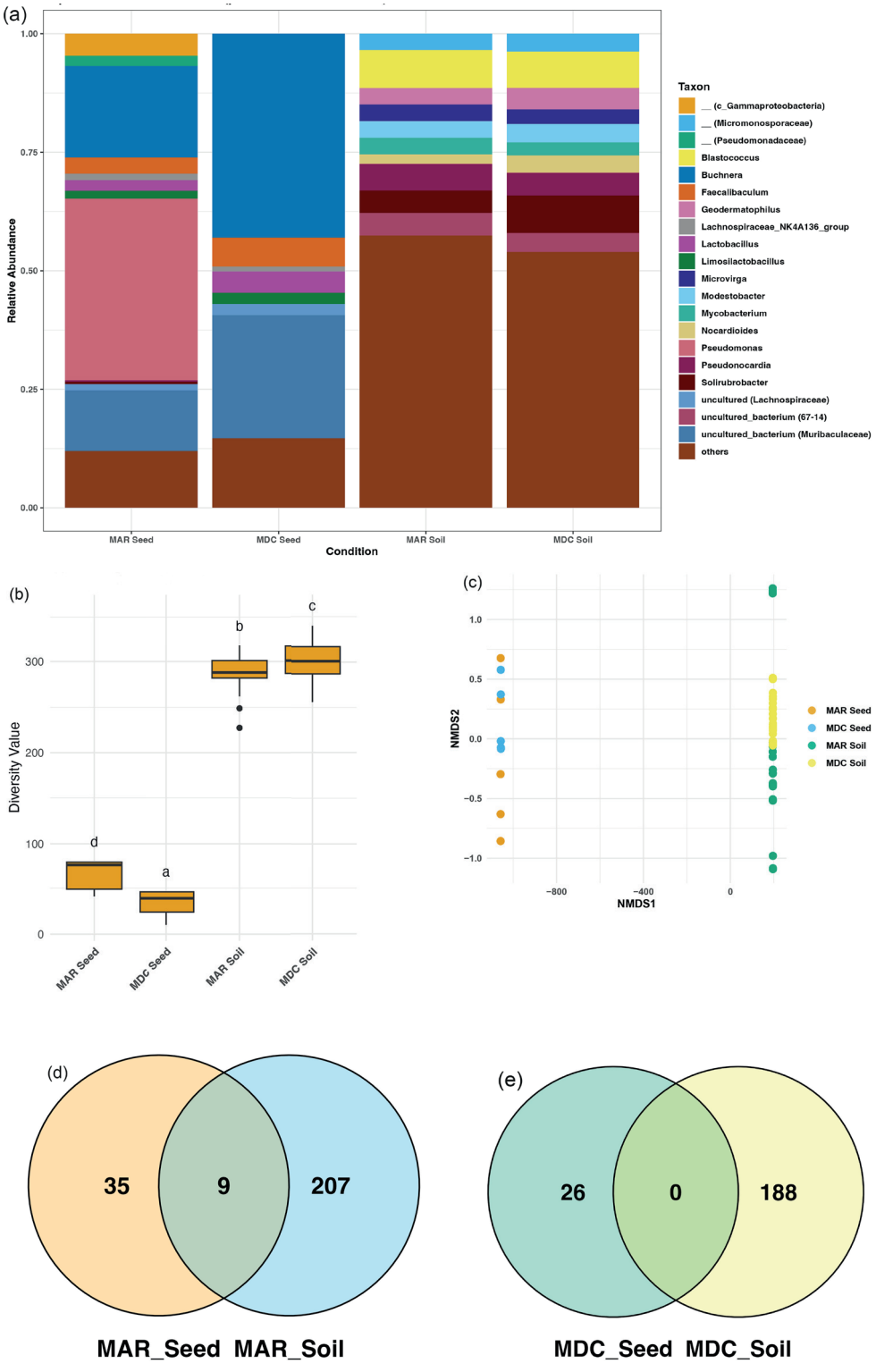

(a)
Relative Abundance
1.00
0.75
0.50
0.25
0.00
MAR Seed
MDC Seed
MAR Soil
MDC Soil
Condition
Taxon
__ (c_Gammaproteobacteria)
__ (Micromonosporaceae)
__ (Pseudomonadaceae)
Blastococcus
Buchnera
Faecalibaculum
Geodermatophilus
Lachnospiraceae_NK4A136_group
Lactobacillus
Limosilactobacillus
Microvirga
Modestobacter
Mycobacterium
Nocardioides
Pseudomonas
Pseudonocardia
Solirubrobacter
uncultured (Lachnospiraceae)
uncultured_bacterium (67-14)
uncultured_bacterium (Muribaculaceae)
others
(b)
Diversity Value
300
200
100
0
a
b
c
d
MAR Seed
MDC Seed
MAR Soil
MDC Soil
(c)
NMDS2
1.0
0.5
0.0
−0.5
−1.0
−800
−400
0
NMDS1
MAR Seed
MDC Seed
MAR Soil
MDC Soil
(d)
35
9
207
MAR_Seed
MAR_Soil
(e)
26
0
188
MDC_Seed
MDC_Soil

Figure 3: Comparison of wild *Arabidopsis thaliana* seed microbiomes (MAR and MDC populations) and their bulk soil bacterial communities. (a) Relative abundance of the ten most dominant bacterial genera (corresponding family indicated in parentheses) in seeds and soil samples. Genera outside the top ten in overall abundance were grouped under "Other" for visualization clarity. Taxa labelled as "uncultured" were identified only by sequencing and cannot be assigned to a formal genus, but are reported at higher taxonomic ranks (e.g., family or class). (b) Alpha diversity (Observed Diversity Index) of seed MDC and MAR and their bulk soil microbiomes. Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$. (c) NMDS plot based on Bray-Curtis dissimilarity showing separation between seed and soil bacterial communities. The ordination was performed in three dimensions ($k = 3$) and was highly stable (stress ≈ 0.0001). Community differences were significant according to PERMANOVA ($R^2 = 0.078$, $F = 1.702$, $p = 0.037$, permutations = 999). (d) Venn diagram showing nine shared genera between MAR seeds and their bulk soil. (e) Venn diagram showing no shared genera between MDC seeds and their bulk soil.

The host growing environment shapes seed microbiome composition, while a conserved core persists across environments

To investigate how the host growing environment influences seed-associated bacterial communities, we compared wild *Arabidopsis thaliana* seeds collected from three distinct locations in Spain (MAR, MDC, and POB populations) with seeds from greenhouse populations (Col-0, L*er*, Bay, Sha) propagated for three generations (G3) under controlled conditions on different substrates (data from Chapter 2). Taxonomic profiles from 16S rRNA gene sequencing revealed that wild and greenhouse *Arabidopsis thaliana* seeds harboured distinct endophytic bacterial communities (Supplementary Fig. 2a). Beta diversity analysis further highlighted a clear separation between wild and greenhouse seed microbiomes. The NMDS ordination provided a highly stable representation of community dissimilarities (stress ≈ 0.0001), indicating that the observed clustering reliably reflects differences in microbial composition. These compositional differences were statistically supported by PERMANOVA ($R^2 = 0.45885$, $F = 2.4495$, $p = 0.001$, permutations = 999), showing that nearly 46% of the variation in seed bacterial communities can be attributed to host environmental conditions. This result suggests that environmental factors, including exposure to soil microbiota, local climatic conditions, and natural plant-microbe interactions, play a dominant role in shaping which bacterial taxa colonize seeds, leading to distinct microbial profiles between wild and greenhouse populations. In contrast, alpha diversity varied among populations, with some exhibiting higher richness than others (Supplementary Fig. 2b), potentially reflecting differences in local environmental conditions or host genotype. Overall, these results demonstrate that the host growth environment strongly shapes both the composition and structure of seed-associated bacterial communities.

Despite these pronounced compositional differences, a conserved set of bacterial genera was consistently detected across wild and greenhouse environments. Grouping greenhouse populations by substrate type (rockwool, potting soil, gamma-sterilized field soil, and non-sterile field soil) and pooling accessions, we focused on bacterial taxa stably associated with seeds in each environment, independent of genotype. Venn diagram analysis revealed the core seed microbiota shared across wild and greenhouse environments (Fig. 4b). A total of 17 out of 77 genera (22.4%) were consistently detected across all populations, defining a conserved "core seed microbiome" of *Arabidopsis thaliana*. These conserved genera were primarily associated with the phyla Firmicutes (10 genera), including *Ligilactobacillus*, *Blautia*, *Faecalibaculum*, *Lactobacillus*, *Limosilactobacillus*, *Lachnospiraceae_NK4A136_group*, *Lachnospiraceae_UCG-006*, and two uncultured taxa from the families *Lachnospiraceae* and *Oscillospiraceae*; and Bacteroidota (5 genera), including an unidentified member of *Muribaculaceae*, *Muribaculum*, *Prevotellaceae_UCG-001*, *Alloprevotella*, and *Alistipes*. Two additional genera were also detected from Campylobacterota (*Helicobacter*) and Deferribacterota (*Mucispirillum*) (Table 2). These results indicate that a subset of bacterial genera consistently colonizes Arabidopsis seeds across diverse growth environments, suggesting that these core endophytes may be stably maintained by the host, potentially through vertical transmission, and could play important roles in seed health, germination, and early seedling development.

Several bacterial genera displayed environment-specific patterns (Fig. 4b). All three wild populations harboured unique genera, with MAR seeds showing the highest number (14 genera, 18.4%), followed by MDC (2 genera, 2.6%) and POB (1 genus, 1.3%). Among greenhouse seeds, only those grown in non-sterile field soil contained unique taxa (2 genera, 2.6%), whereas seeds cultivated in low-microbial-diversity substrates such as rockwool, potting soil, or sterilized field soil contained no unique genera. These patterns suggest that environmental microbial exposure strongly influences the presence of unique seed-associated taxa, with natural soils supporting a more diverse and distinctive microbiome compared to controlled or low-diversity greenhouse substrates. Examples of environment-specific taxa include *Rikenellaceae_RC9_gut_group* and *Parabacteroides*, which were detected exclusively in seeds from greenhouse plants grown in non-sterile field soil. In the wild populations, *Prevotella_9* was unique to POB seeds, while *Pantoea* and *Rickettsiella* were found only in MDC seeds. The largest set of unique genera occurred in MAR seeds, including *Pseudomonas*, *Cellulomonas*, *Pseudonocardia*, *Conexibacter*, *Solirubrobacter*, *Clavibacter*, *Roseomonas*, and *Microbacterium*. Additional genera were shared by specific pairs of wild populations but not with greenhouse populations. For example, *Buchnera*, *Escherichia–Shigella*, and *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium*

were detected in MAR and MDC seeds or in MAR and POB seeds. Conversely, several genera were shared exclusively among greenhouse populations, but not with wild populations, such as *Colidextribacter* and *Candidatus_Arthromitus*, along with several unidentified taxa from the families *Oscillospiraceae*, *Muribaculaceae*, *Clostridiaceae*, and *Desulfovibrionaceae* (Supplementary Table 6). Together, these findings show that a conserved core Arabidopsis seed microbiome persists across environments and local environmental conditions contribute to the presence of unique, environment-specific bacterial taxa. This highlights the combined influence of host environment and substrate microbial diversity in structuring the *Arabidopsis thaliana* seed microbiome.

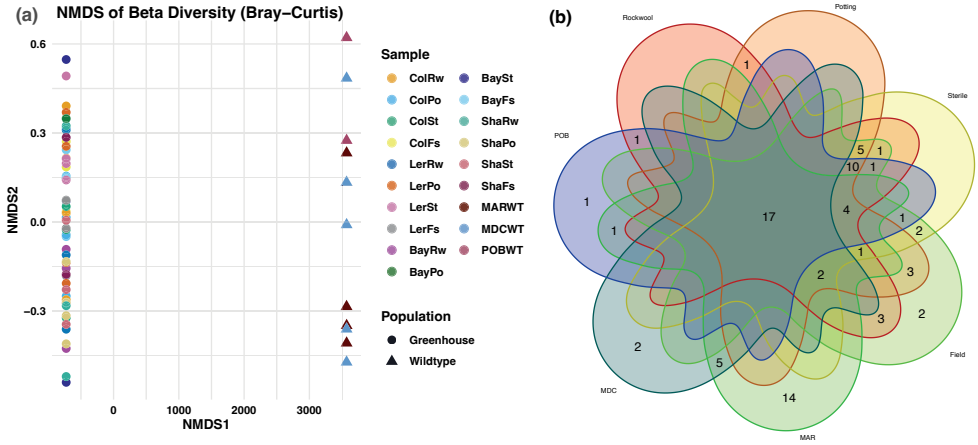


Figure 4: Comparison of seed microbiome composition between wild and greenhouse-grown *Arabidopsis thaliana*. Wild populations include MAR, MDC, and POB, while greenhouse populations consist of four accessions (Col-0, L*er*, Bay, and Sha) propagated for three generations on different substrates: rockwool (Rw), commercial potting substrate (Po), gamma-sterilized field soil (St), and non-sterile field soil (Fs). (a) Beta diversity of seed-associated bacterial communities from wild populations (MAR, MDC, POB) and greenhouse-grown populations visualized by non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarity. NMDS was performed using three dimensions (k = 3), and the ordination was highly stable (stress ≈ 0.0001). Community composition differed significantly among groups, as supported by PERMANOVA (R^2 = 0.45885, F = 2.4495, p = 0.001, permutations = 999). (b) Venn diagrams showing shared and unique seed-associated bacterial genera at the genus level among wild populations and greenhouse populations grouped by substrate type. Greenhouse accessions were pooled by substrate to identify bacterial taxa consistently associated with seeds within each growth environment. Genera shared across all populations represent the Arabidopsis core seed microbiome.

Table 2: Core *Arabidopsis thaliana* seed microbiome across wild and greenhouse environments. Bacterial endophytic genera consistently detected in seeds from all populations, including three wild populations (MDC, MAR, and POB) and greenhouse-grown plants which were grouped by substrate type (rockwool, potting soil, gamma-sterilized field soil, and non-sterile field soil), with accessions pooled for analysis. Taxa were identified using 16S rRNA gene amplicon sequencing and are reported at five taxonomic ranks: phylum, class, order, family, and genus.

No.	Phylum	Class	Order	Family	Genus
1	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*uncultured_bacterium*
2	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	*Muribaculum*
3	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotellaceae_UCG-001*
4	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Alloprevotella*
5	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Alistipes*
6	Campylobacterota	*Campylobacteria*	*Campylobacterales*	*Helicobacteraceae*	*Helicobacter*
7	Deferribacterota	*Deferribacteres*	*Deferribacterales*	*Deferribacteraceae*	*Mucispirillum*
8	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Faecalibaculum*
9	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*
10	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Limosilactobacillus*
11	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*uncultured*
12	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*A2*
13	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*uncultured*
14	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_NK4A136_group*
15	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Ligilactobacillus*
16	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-006*
17	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Blautia*

Population-specific cultivable endophyte communities in wild *Arabidopsis thaliana* seeds

To characterize the cultivable seed endophytic bacterial communities associated with wild *Arabidopsis thaliana* populations, we isolated seed endophytic bacteria from three wild *Arabidopsis thaliana* populations (MAR, MDC, and POB). Recovering cultivable isolates allowed us to obtain viable seed-associated bacteria for taxonomic identification and subsequent assessment of their potential beneficial roles in promoting early plant growth. Bacterial endophytes were isolated using LB, R2A, TSA, and PDA media incubated at 20°C and identified by Sanger sequencing of the 16S rRNA gene. No bacterial colonies were recovered from POB seeds, which may reflect a low abundance of cultivable bacteria or population-specific differences in microbiome composition. In contrast, nine bacterial strains were recovered from each of the MAR and MDC populations, with all isolates showing >97% BLAST sequence similarity to reference taxa (Table 3; Supplementary Fig. 3-4). The cultivable endophyte communities differed markedly between MAR and MDC seeds. MDC isolates were primarily Firmicutes (5 strains, 56%), Actinomycetota (3 strains, 33%), and Pseudomonadota (1 strain, 11%). In contrast, the MAR population was dominated by Pseudomonadota (6 strains, 67%), with the remaining isolates assigned to Firmicutes, Bacteroidota, and Actinomycetota (1 strain each, 11%) (Table 3; Supplementary Fig. 3-4). Colony morphology also varied across populations and growth media, reflecting the diversity of cultivable seed-associated bacteria (Fig. 5). These population-specific patterns align with the differentiation observed in the 16S rRNA amplicon sequencing profiles. Only one strain, *Peribacillus frigoritolerans* DSM 8801, was shared between the MAR and MDC populations, represented by isolates MAR22 and MDC39, respectively (Table 3; Supplementary Fig. 3-4). The presence of this strain in two geographically distinct populations suggests that some seed endophytes may be stably maintained across environments. However, the recovery of only a single shared strain underscores that the cultivable seed microbiomes of MAR and MDC are highly distinct, with most taxa being population-specific. Notably, none of the cultivable genera recovered from wild seeds were detected in the 16S rRNA amplicon sequencing dataset. Together, these findings indicate that differences in local environmental conditions may contribute to shaping population-specific cultivable seed endophyte communities in wild *Arabidopsis thaliana*.

Table 3: Bacterial endophytes isolated from wild *Arabidopsis thaliana* seeds. Bacterial strains were isolated from seeds of the Marjaliza (MAR) and Moral de Calatrava (MDC) populations and identified by Sanger sequencing of the 16S rRNA gene.

Code	Population	Phylum	Strains	BLAST similarity (%)
MDC18	Moral de Calatrava	Firmicutes	*Bacillus mobilis strain MCCC 1A05942*	100.00
MDC11	Moral de Calatrava	Firmicutes	*Bacillus wiedmannii strain FSL W8-0169*	100.00
MDC31	Moral de Calatrava	Firmicutes	*Neobacillus drentensis strain NBRC 102427*	99.70
MDC39	Moral de Calatrava	Firmicutes	*Peribacillus frigoritolerans strain DSM 8801*	99.93
MDC19	Moral de Calatrava	Firmicutes	*Priestia aryabhattai B8W22*	100.00
MDC40	Moral de Calatrava	Actinomycetota	Leifsonia poae strain VKM Ac-1401	99.93
MDC35	Moral de Calatrava	Actinomycetota	Leifsonia shinshuensis strain DB 102	99.00
MDC16	Moral de Calatrava	Actinomycetota	*Rathayibacter caricis strain VKM Ac-1799*	99.78
MDC34	Moral de Calatrava	Pseudomonadota	*Sphingomonas insulae strain DS-28*	98.68
MAR2	Marjaliza	Pseudomonadota	Pseudomonas viridiflava strain CECT 458	99.92
MAR8	Marjaliza	Pseudomonadota	*Sphingomonas faeni* strain MA-olki	98.81
MAR13	Marjaliza	Pseudomonadota	*Pseudomonas viridiflava* strain ATCC 13223	99.40
MAR15	Marjaliza	Pseudomonadota	*Pseudomonas lutea* strain OK2	99.24
MAR61	Marjaliza	Pseudomonadota	*Sphingomonas endophytica* strain YIM 65583	97.88
MAR82	Marjaliza	Pseudomonadota	*Methylobacterium cerastii* strain C15	100.00
MAR22	Marjaliza	Firmicutes	*Peribacillus frigoritolerans* strain DSM 8801	98.86
MAR24	Marjaliza	Bacteroidota	*Hymenobacter metalli* strain A2-91	96.90
MAR27	Marjaliza	Actinomycetota	*Frigoribacterium faeni* strain 801	98.46

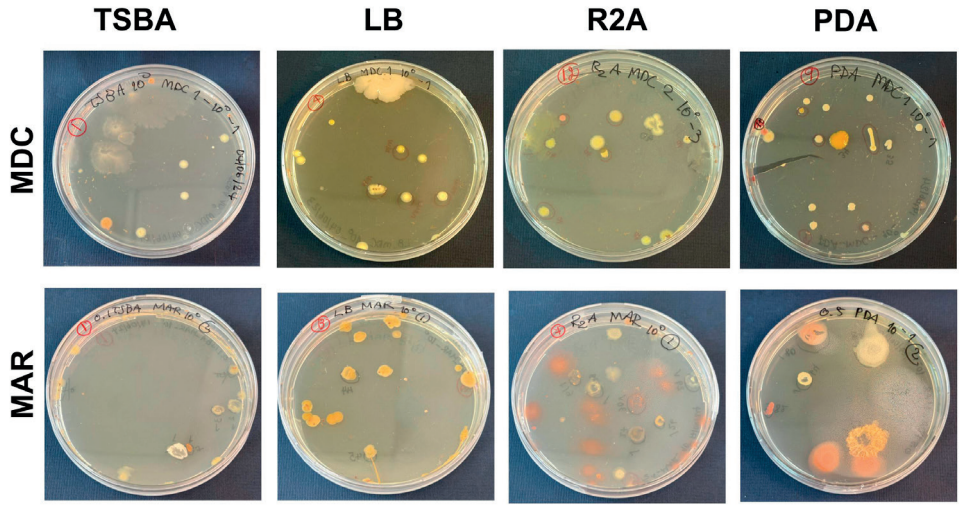


Figure 5: Cultivable seed-associated bacteria from wild *Arabidopsis thaliana* seeds. Representative petri dishes showing bacterial colonies directly isolated from undiluted seed suspensions of the MAR and MDC populations. Each dish contains a diverse assemblage of cultivable endophytes present in the seeds, prior to individual colony purification. No colonies were recovered from the POB population. Different media (LB, PDA, R2A, and TSBA) were used to capture a broad range of bacterial taxa.

Seed-associated bacterial volatiles promote Arabidopsis germination under osmotic stress

Since the strain *Peribacillus frigoritolerans* was the only bacterium shared between the MAR and MDC wild populations in our isolate collection (Table 3), we assessed whether volatiles produced by this seed endophytic bacterium influence *Arabidopsis thaliana* seed germination. To this end, L*er* seeds were exposed to bacterial volatiles by placing bacterial cultures on separate agar plates within the same sealed system, allowing diffusion of volatiles to the seeds without physical contact. Two independently isolated representatives of *Peribacillus frigoritolerans* (strains MDC39 and MAR22; Table 3; Supplementary Figs. 3 and 4) were used in the assay. An additional bacterial strain, *Pantoea agglomerans* E44, isolated from wild cabbage seeds, was included as a positive control because its volatiles have previously been shown to promote *Arabidopsis* seed germination (Chapter 3). Germination was assessed under non-stress and osmotic stress (500 mM D-mannitol) conditions.

Exposure to bacterial volatiles had a marked effect on Arabidopsis seed germination, particularly under osmotic stress. Under non-stress conditions, seeds germinated efficiently across all treatments, and no substantial differences were observed between seeds exposed to bacterial volatiles and the non-bacterial

control (Fig. 6a and 6b). In contrast, severe osmotic stress (500 mM D-mannitol) strongly reduced germination in both the presence and absence of bacterial volatiles, confirming that this treatment effectively inhibited seed germination (Fig. 6a and 6b).

Under osmotic stress, Arabidopsis seeds exposed to volatiles from the endophytic strain *Peribacillus frigoritolerans* (MDC39 or MAR22) germinated significantly better than seeds exposed to non-bacterial volatiles (P-buffer) or *Pantoea agglomerans* E44. In the single-plate assay, germination increased from 40% in controls to 51% with *P. frigoritolerans* volatiles (Fig. 6a), and in the double-plate setup, where volatile concentration was higher, germination reached 63-70% in the presence of the bacterial volatiles (Fig. 6b). No significant differences were observed between the two *P. frigoritolerans* strains, indicating similar effects on germination. Germination rate (T50) analysis only showed a significant difference in the double-plate setup, seeds exposed to MDC39 and MAR22 volatiles exhibited significantly lower T50 values, indicating faster germination compared to E44 or P-buffer (Supplemental Fig. 5). No significant differences were detected between MDC39 and MAR22 (Supplemental Fig. 5). These results suggest that volatiles from *P. frigoritolerans* accelerate Arabidopsis seed germination and enhance tolerance to osmotic stress.

Comparing the single- and double-plate setups directly under osmotic stress revealed that seeds in the double-plate setup consistently displayed higher germination percentages across all treatments, including the P-buffer control (Supplemental Fig. 6). This indicates that increased volatile abundance, whether originating from bacteria or the growth medium, enhances seed germination under osmotic stress. Analysis of T50 further indicated that only seeds exposed to *P. frigoritolerans* volatiles germinated faster in the double-plate setup than in the single-plate setup (Supplemental Fig. 6), highlighting a dose-dependent effect of bacterial volatiles on germination rate. Together, these findings suggest that seed-associated endophytic bacteria can enhance *Arabidopsis thaliana* germination under osmotic stress conditions, indicating a potential role for these microbes in supporting early plant development under stress. These results highlight the potential of seed-associated bacteria as candidates for future strategies aimed at improving crop performance under adverse environmental conditions.

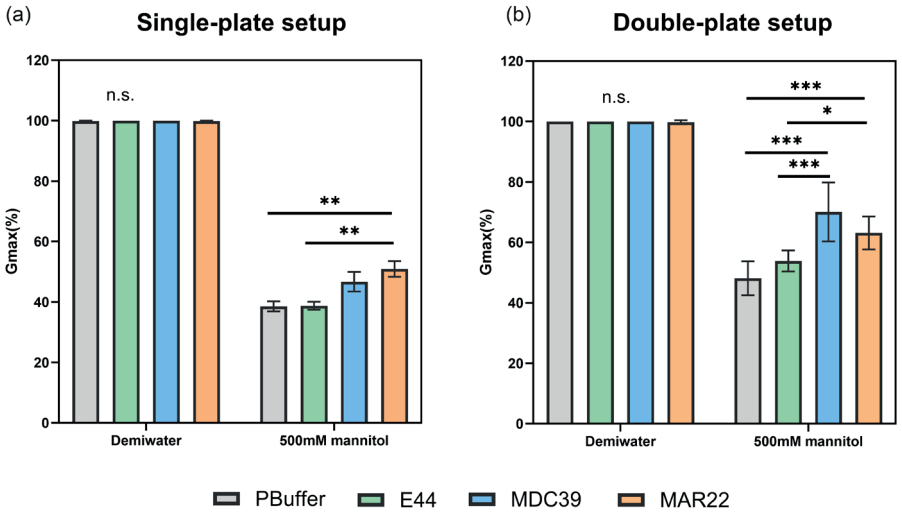


Figure 6: Germination of *Arabidopsis thaliana* seeds exposed to bacterial volatiles under non-stress and osmotic stress conditions. Seeds were exposed to volatiles emitted by the seed endophytic strains *Peribacillus frigoritolerans* MDC39 and MAR22, the positive control *Pantoea agglomerans* E44, or phosphate buffer (non-volatile negative control). Germination was assessed under non-stress (demineralized water) and osmotic stress (500 mM D-mannitol) conditions using either a single-plate or double-plate volatile exposure setup. (a-b) Germination percentage (Gmax) for single-plate and double-plate setups, respectively. Data represent mean ± SE; statistical significance was determined by ANOVA followed by Tukey's post hoc test. Significance is indicated as follows: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

DISCUSSION

The host growth environment is a key factor shaping the assembly of seed-associated microbial communities (Rochefort et al., 2019a; Tabassum et al., 2024). In this study, we compared seeds from three wild *Arabidopsis thaliana* populations collected from Spain (POB, MAR, and MDC) with accessions propagated for three generations under controlled greenhouse conditions. Our results demonstrate that the host growth environment strongly shapes seed microbiome composition, resulting in distinct microbial communities across populations. Despite this pronounced environmental influence, we identified a conserved core seed microbiome shared across wild and greenhouse-grown plants, indicating that a subset of bacterial taxa is consistently maintained in seeds across contrasting growth conditions. In addition, wild seeds harboured diverse cultivable endophytic bacteria, including *Peribacillus frigoritolerans*, a strain shared by two of the wild populations that have been investigated. This bacterium showed the potential to promote plant growth by enhancing Arabidopsis seed germination under osmotic stress through the emission of volatile organic compounds (VOCs).

When plants grow in natural environments, their seeds often harbour uniquely diverse endophytic bacterial communities due to the highly variable and complex conditions of these ecosystems (Liang et al., 2021; Wassermann et al., 2019). Consistent with this, the three wild *Arabidopsis thaliana* populations examined here harboured distinct seed microbiomes, indicating strong site-specific influences on microbial recruitment (Fig. 1). The frequent dominance of genera such as *Pseudomonas and Pantoea* (Fig. 1b; Supplementary Table 2) agrees with previous reports from wild plant species (Liu et al., 2022), where seed-associated communities vary across habitats but often include environmentally adaptable taxa. These population-specific microbiomes highlight the strong influence of local environmental conditions on microbial recruitment into seeds.

Comparing bacterial communities of seeds and their corresponding bulk soils supports horizontal transmission of microbes from soil to seeds, although the contribution appears relatively small. Only seeds from the MAR population shared a subset of bacterial genera with their surrounding soil, representing just 3.6% of the seed-associated microbiota. These shared taxa represent *Pseudomonas*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium,* and *Pseudonocardia* species (Fig. 3d; Supplementary Table 4). Soil and seed overlap involving some of these genera has also been reported in drought-tolerant legumes from Namibia, where *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* and *Pseudonocardia* were shared between seeds and their corresponding bulk soils (Mataranyika et al., 2024). *Pseudomonas* species are commonly widespread in soil and rhizosphere environments (Lopes et al., 2018) and are frequently identified as seed endophytes (Truyens et al., 2015), supporting the idea that soil contributes horizontally transmitted seed-colonizing bacteria (Chen et al., 2023; Frank et al., 2017).

Beyond soil, the majority of seed-associated bacteria are likely transmitted vertically from the maternal plant (Sanz-Puente et al., 2025; Sulesky-Grieb et al., 2024) or acquired from aboveground sources, such as insects or pollinators that interact with flowers and influence seed development (Prado et al., 2020). One such example is *Buchnera*, a genus typically associated with aphids (Douglas, 2003), which was detected only in seeds from the MAR and MDC populations (Fig. 4b; Supplementary Table 6). Although this aphid-associated bacterium rarely occurs naturally in plants, its presence in wild Arabidopsis seeds may indicate transfer from insect-associated microbial communities (Chrostek et al., 2017; Pons et al., 2019). However, the route by which *Buchnera* becomes associated with seeds remains unclear, and further studies are needed to determine whether insects contribute to its transmission into seeds.

The lack of overlap between seed and soil microbiomes may reflect the selective filtering that soil microbes undergo to colonize seeds, as microbes are first established in roots, then passed through stems and other plant tissues before finally reaching seeds (Mayer et al., 2025; Rodríguez et al., 2018). Additionally, because our study sampled bulk soil rather than rhizosphere soil, the contribution of plant-recruited microbes may be underestimated. While many rhizosphere taxa originate from the surrounding bulk soil and may also be present there at lower abundance, the rhizosphere represents the actively selected microbial fraction shaped by root exudates and plant-microbe interactions. As such, rhizosphere sampling would likely provide a more accurate representation of potential seed-colonizing bacteria (Beauregard, 2015; Santoyo, 2022; Yang et al., 2025a). Supporting this view, Rocha et al. (2025) demonstrated that rhizosphere communities supplied genera such as *Bacillus*, *Streptomyces,* and *Paenibacillus* to progeny seeds of lima bean. Thus, the limited overlap observed in populations such as MDC may reflect both strong ecological filtering and the lack of rhizosphere sampling. The limited transfer of soil-derived bacteria into internal plant tissues is consistent with our alpha and beta diversity analyses. Wild Arabidopsis seeds harboured dramatically lower bacterial diversity than their corresponding bulk soils, and beta diversity analyses showed a clear separation between seed and soil communities (Fig.3b-c), consistent with a strong selective bottleneck during seed colonization. Similar patterns have been reported across plant species, Styrian oil pumpkin seeds exhibiting significantly lower diversity and distinct composition compared to soil and rhizosphere communities (Kusstatscher et al., 2021). Lima bean seeds were dominated by *Acinetobacter* and *Pseudomonas* while their corresponding soil was enriched in *Bacillus* (Rocha et al., 2025), and tomato seeds harboured selective, low-diversity communities dominated by *Burkholderiaceae* and *Pseudomonadaceae*, contrasting sharply with the highly diverse soil microbiota dominated by *Acidobacteria* and *Planctomycetes* (Bergna et al., 2018). Together, these results suggest that the limited soil contribution to seed microbiomes is driven by strong, host-mediated selective filtering, whereby only a small subset of soil microbes that are compatible with plant tissues can successfully colonize and persist in seeds.

Despite big population-specific differences, we identified a limited core seed microbiome in the wild populations, representing 29.5% of the total detected genera (Fig. 2). The core microbiome was mainly composed of Firmicutes (e.g., *Faecalibaculum*, *Lactobacillus*, *Blautia*, *Carnobacterium*), Bacteroidota (e.g., *Muribaculum*, *Alistipes*), Deferribacterota (*Mucispirillum*), and Campylobacterota (*Helicobacter*) (Table 1). In addition to the core taxa, each population harboured unique bacterial genera, with a high proportion that collectively accounted for 51% of the total detected genera (Fig. 2). The relatively small size of the wild

seed core microbiome, together with the high proportion of population-specific unique taxa, likely reflects strong host-driven filtering and microbial competition occurring in natural ecology (Pérez-Lorente et al., 2024; Wani et al., 2022). Environmental variability and horizontal acquisition from sources such as soil, insects, pollinators, and neighbouring plants further generate population-specific communities, limiting the taxa consistently maintained across sites (Eyre et al., 2019; Mészárošová et al., 2024). Limited core microbiomes are common in wild plants. In native alpine species, seeds harboured highly diverse, species-specific microbial communities, with an exceptionally limited core (3% of taxa) composed of Proteobacteria (*Tatumella*, *Pseudomonas*, *Serratia*, *Burkholderia*) and Firmicutes (*Bacillus*) (Wassermann et al., 2019). Similarly, wild Panax seeds contained a small core microbiome (20% of taxa) dominated by Proteobacteria (*Halomonas*, *Pseudomonas*) (Liu et al., 2022). Although the genera in our Arabidopsis core differ from those in these studies, the overlap at the phylum level, particularly Firmicutes, highlights a conserved higher-level structure of seed microbiomes. The lack of genus-level overlap underscores the species-specific nature of wild seed microbiomes, yet the persistence of a core across ecologically distinct populations suggests that certain microbial lineages are consistently maintained. Such conservation likely reflects their potential adaptability to colonize seed tissue and functional importance for host fitness (War et al., 2023).

Comparison between wild and greenhouse seeds further highlights the strong influence of the growth environment on seed microbiome assembly. Seeds produced under greenhouse conditions harboured bacterial communities that were clearly distinct from those of wild populations, as evidenced by a clear separation in beta diversity (Fig. 4a). Consistent with our findings, Bergna et al. (2018) showed that tomato seeds produced under greenhouse conditions harboured microbial communities that were reduced in diversity and clearly distinct from those of field-grown plants. The work by Dissanayakalage et al. (2025) provides a complementary example in lucerne (*Medicago sativa*), showing that domestication (or controlled cultivation) reduces seed microbiome diversity, while wild relatives maintain broader and more functionally diverse communities. In our study, greenhouse populations generally lacked population-specific taxa, with most bacterial genera shared across populations. Only populations propagated in microbially diverse field soil retained a small set of environment-specific taxa (Fig. 4b). These patterns indicate that restricted greenhouse conditions constrain microbial recruitment and diversification due to limited exposure to environmental microbial sources (Legein et al., 2022). Greenhouse-specific taxa included *Rikenellaceae_RC9_gut_group*, *Parabacteroides*, *Colidextribacter* and *Candidatus Arthromitus*, (Supplementary Table 6), which are associated with animal or human gut microbiomes rather than known

plant-beneficial functions (Di Vincenzo et al., 2024; Liu et al., 2025a; Million et al., 2018; Sebastià et al., 2024). Their exclusive occurrence in greenhouse seeds suggests that human cultivation practices, including irrigation systems, handling, and repeated plant contact, may facilitate the introduction of human-associated taxa into greenhouse populations (Abdelkader et al., 2025; Abdullaeva et al., 2021).

In contrast, wild seeds harboured highly distinct, population-specific microbial assemblages, with numerous unique taxa absent from greenhouse seeds (Fig. 4b; Supplementary Table 6). A closer look at these wild-specific taxa shows that many of them are well-known plant growth-promoting bacteria. For example, *Pseudomonas* (Sah et al., 2021) and *Pantoea* (Dastager et al., 2009), which contribute to nutrient solubilization, indole-3-acetic acid (IAA) production, siderophore production, and biocontrol. Other beneficial taxa included *Cellulomonas,* reported to enhance seed germination through cellulase production (Smirnova & Sadanov, 2019), *Microbacterium*, which promotes seed germination and suppresses grey mold via volatile organic compounds (Suárez-Estrella et al., 2023), and nitrogen-fixing members of the *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (Idowu et al., 2024). Additionally, several taxa commonly associated with insect guts or insect endosymbionts, including *Rickettsiella* (Iasur-Kruh et al., 2013), *Buchnera* (Shigenobu et al., 2000), and *Escherichia-Shigella* (Young et al., 2020) were detected exclusively in wild seeds, highlighting the contribution of insect-mediated horizontal transmission to seed microbiome assembly in natural environments. These results emphasize wild seeds as reservoirs of beneficial, stress-resilient microbes that could be harnessed to restore microbial diversity in breeding and crop improvement programs. Overall, seed microbiomes reflect the host's growth environment, with natural environments promoting higher microbial diversity and functional potential through extensive opportunities for horizontal transmission from soil, insects, and other (a)biotic interactions, whereas controlled greenhouse conditions constrain microbial recruitment and favour limited communities that may include non-beneficial or human-associated taxa.

Despite pronounced differences in seed microbial communities between wild and greenhouse populations, we identified a consistent Arabidopsis seed core microbiome shared across all populations, representing 22.4% of detected taxa (Fig. 4b; Table 2). The persistence of this small, conserved core across ecologically distinct wild populations, multiple *Arabidopsis thaliana* accessions, and greenhouse-grown seeds propagated for several generations suggests that these taxa are maintained despite contrasting environmental conditions through species-specific host filtering and potential vertical transmission from

maternal tissues (Dissanayakalage et al., 2025; Wu et al., 2024; Zeng et al., 2023). Evidence from other plant species also supports the existence of conserved seed-associated microbial communities. Moroccan argan seeds shared genera such as *Pseudomonas*, *Bacillus*, and *Aspergillus* across locations (Sohaib et al., 2024), while rice seeds maintained core genera including *Pantoea*, *Pseudomonas*, *Methylobacterium*, *Sphingomonas*, and *Xanthomonas* across geographically distinct locations (Zhou et al., 2020). At a broader scale, *Pantoea* and *Pseudomonas* were identified as dominant members of a global seed microbiome across 50 plant species (Simonin et al., 2022) and were consistently detected in seeds from 17 plant species (Johnston-Monje et al., 2021). Although *Pantoea* and *Pseudomonas* are frequently reported as members of seed core microbiomes across plant species and environments, they were not part of the Arabidopsis core identified in our study but were detected in wild seed samples (Supplementary Table 6), indicating their presence is driven by environmental exposure. Together, these observations indicate that Arabidopsis consistently maintains a small, conserved, host-selected core adapted to the seed niche, demonstrating strong species-specific filtering and potential vertical transmission across genotypes and environments.

Cultivable bacterial endophytes isolated from wild *Arabidopsis thaliana* seeds revealed pronounced population-specific differences that complement patterns observed in the 16S rRNA sequencing data. Nine strains were recovered from the MAR and MDC populations each, of which only one strain, *Peribacillus frigoritolerans* DSM 8801, a well-documented plant growth-promoting and antibiotic bacterium (Mazuecos-Aguilera et al., 2025; Świątczak et al., 2024), was shared between the two populations (Table 3). Remarkably, *P. frigoritolerans* was not detected in the 16S rRNA amplicon dataset, likely because its low relative abundance fell below sequencing detection limits or was removed during bioinformatic filtering of rare reads (Breitenwieser et al., 2020; Nikodemova et al., 2023). The detection sensitivity of microbial species might have been affected by the high proportion of plant-derived DNA that could have masked the microbial 16S amplification (Lefèvre et al., 2020). This highlights the ability of cultivation-based approaches to recover rare or low-abundance taxa overlooked by molecular profiling (Shade et al., 2012). Beyond the shared *P. frigoritolerans* strain, several isolates recovered from the MDC population (Table 3), including *Bacillus mobilis*, *Bacillus wiedmannii*, *Neobacillus drentensis*, *Priestia aryabhattai*, *Leifsonia poae*, and *Leifsonia shinshuensis*, have been reported to exhibit plant growth promoting, antimicrobial, or biocontrol activities in various plant species (Deng et al., 2022; Hernández-Pacheco et al., 2021; Jiang et al., 2022; Kumar et al., 2025; Poovarasan et al., 2013; Wu et al., 2019). Similarly, isolates from the MAR population, such as *Pseudomonas lutea* and *Frigoribacterium faeni* have previously been described as plant growth-promoting bacteria (Kwak et al.,

2016; Pereira & Castro, 2014). Although some endophytic strains recovered in this study, including *Sphingomonas endophytica* and *Methylobacterium cerastii*, have not yet been directly linked to plant growth promotion, their genera are well known for diverse metabolic capabilities and beneficial plant-microbe interactions (Asaf et al., 2020; Ehinmitan et al., 2025). Moreover, the cultivable *Pseudomonas* strains identified in the MAR populations were consistent with our 16S rRNA amplicon sequencing data, which showed a high relative abundance of the genus Pseudomonas in MAR populations (Supplementary Table 2). In contrast, no overlap was observed between cultivable strains isolated from wild populations and taxa detected in the greenhouse amplicon sequencing data (Supplementary Table 2). Together, these findings underscore wild Arabidopsis seeds as reservoirs of functionally diverse, population-specific endophytes with promising potential for future microbiome-driven applications, which represent microbial diversity that is likely underrepresented in greenhouse-grown and domesticated plant systems.

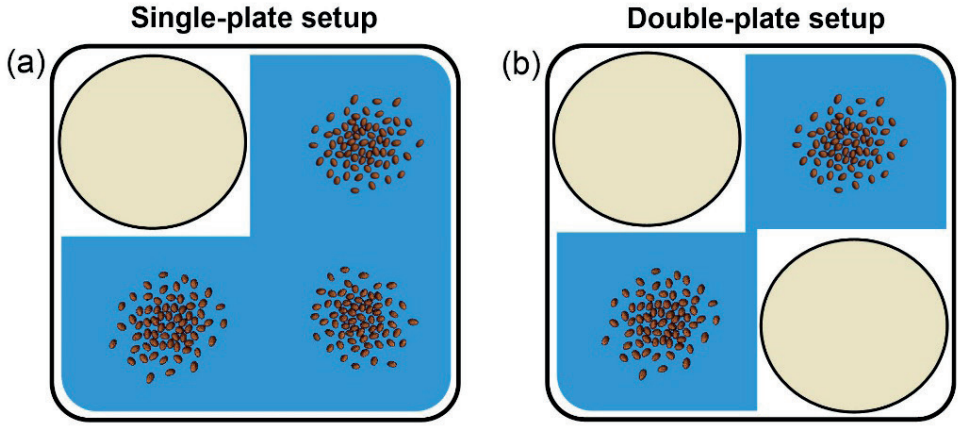
VOCs emitted by plant growth-promoting bacteria serve as crucial signals in plant-microbe interactions (Fincheira et al., 2021). To investigate the functional role of the shared isolate *Peribacillus frigoritolerans*, we assessed the effect of VOCs on *Arabidopsis thaliana* seed germination under osmotic stress. Seeds exposed to VOCs emitted by *P. frigoritolerans* exhibited significantly higher germination percentage compared with the negative control (P-buffer with no bacterial VOCs) and the bacterial positive control *Pantoea agglomerans* E44 (Fig. 6). Remarkably, *P. agglomerans* E44, isolated from wild cabbage seeds, is known to promote seed germination and seedling growth in Arabidopsis (Chapter 3) and cabbage through VOC production (Tyc et al., 2020), yet its effects were surpassed by those of *P. frigoritolerans* under osmotic stress. These results indicate that VOCs emitted by *P. frigoritolerans* can effectively alleviate osmotic stress during early plant development and may confer stronger stress-protective effects than other beneficial seed-associated bacteria. One possible explanation for this enhanced effect is host specificity. *P. frigoritolerans* was isolated from wild Arabidopsis seeds and may therefore be better adapted to interact with Arabidopsis physiology, including the L*er* accession used in this study. Such host-adapted endophytes may produce VOCs that more effectively align with host signalling pathways, resulting in enhanced germination under stress conditions. In a double-plate setup with higher volatile concentration, seeds showed even higher germination percentages and faster germination compared with all other treatments (Supplementary Fig. 6). Interestingly, the P-buffer control also showed increased germination in the double-plate setup compared with the single-plate configuration (Supplementary Fig. 6), suggesting that part of this response may be attributable to volatiles released from the sterile TSBA medium

itself. Previous studies have reported that uninoculated culture media can emit VOCs (Ossowicki et al., 2017; Tyc et al., 2020), although there is currently no direct evidence that medium-derived volatiles alone promote plant growth. This highlights the importance of accounting for background VOCs when interpreting bacterial VOC-mediated effects in plant interaction assays. While *P. frigoritolerans* is recognized as a plant growth-promoting bacterium (Yang et al., 2025b), its VOC-mediated effects on seed germination have not previously been reported. Nevertheless, several studies support its broader beneficial potential. Seed biopriming with *P. frigoritolerans* enhanced tomato seed vigour indices by up to 58% (Pagli et al., 2025). Strain T7-IITJ improved germination of wheat and foxtail millet under drought, while promoting root and shoot growth of Arabidopsis in drought-stress via nutrient solubilization, auxin production, and upregulation of photosynthesis and stress-related genes (Marik et al., 2024). In addition, *P. frigoritolerans* produces nematicidal metabolites like koranimine, supporting its role in plant protection (Montecillo & Bae, 2022). More broadly, numerous studies have demonstrated that bacterial VOCs regulate seed germination, seedling growth, and stress tolerance. Ketone-dominated VOCs released by *Bacillus* sp. BCT9 significantly stimulated lettuce seed germination (Fincheira et al., 2017), while acetoin and 2,3-butanediol emitted by *Bacillus mojavensis, Bacillus subtilis and Enterobacter cloacae* promote Arabidopsis growth (Rath et al., 2018; Ryu et al., 2003). Similarly, VOC blends from *Burkholderia ambifaria* and *Pseudomonas pseudoalcaligenes*, including dimethyl disulfide and 2,3-butanediol, enhance plant growth and stress tolerance across multiple species (Groenhagen et al., 2013; Yasmin et al., 2021). Taken together, our findings demonstrate that the seed-associated endophyte *Peribacillus frigoritolerans* enhances *Arabidopsis thaliana* seed germination under osmotic stress via VOC-mediated mechanisms. This underscores the ecological significance of VOC-producing seed endophytes in promoting early plant establishment under adverse conditions and highlights their potential for application in sustainable agriculture. Future work should focus on identifying the VOCs responsible for these effects, validating their individual roles, and assessing the efficacy of *P. frigoritolerans* as a seed coating or bio-priming agent for economically important crops such as tomato and lettuce.

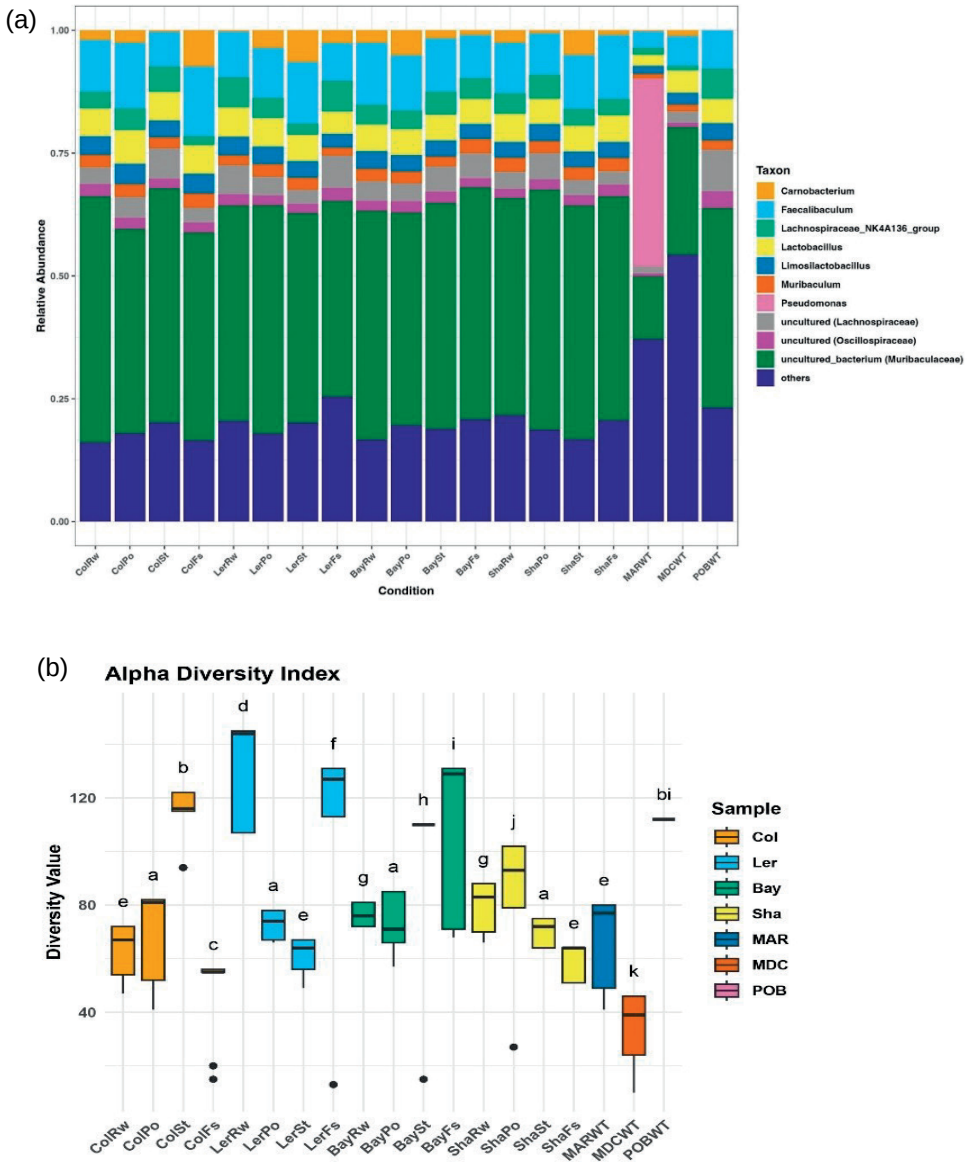
In summary, our study demonstrates that *Arabidopsis thaliana* seed microbiomes are strongly shaped by the host growth environment, with wild populations harbouring highly diverse, population-specific microbial communities, whereas greenhouse conditions constrain diversity and favour limited, non-beneficial taxa. Despite these environmental effects, a small but conserved core microbiome persists across all populations, highlighting host-specific filtering and potential vertical transmission in maintaining key bacterial lineages. Wild seeds also serve as reservoirs of beneficial endophytes, including *Peribacillus*

frigoritolerans, which promotes germination under osmotic stress via VOC emission, emphasizing the ecological and functional importance of host-adapted microbes. These findings underscore the dual influence of environment and host selection in shaping seed microbiomes and highlight the potential of wild seed microbiomes as sources of stress-resilient, growth-promoting bacteria that may be lost during breeding and greenhouse cultivation. Future work should focus on identifying key functional taxa, elucidating their mechanisms, and developing microbiome-based application strategies, such as seed coating or bio-priming, to sustainably enhance crop resilience, early establishment, and productivity under environmental stress.

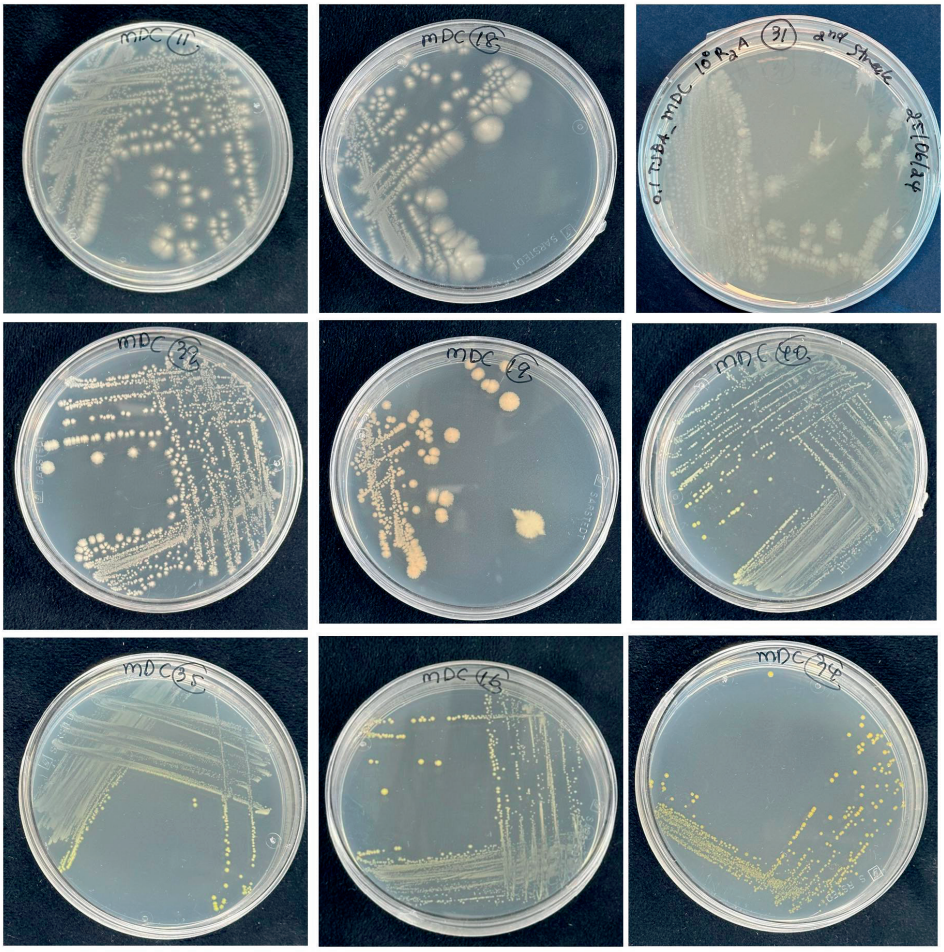
SUPPLEMENTARY FIGURES



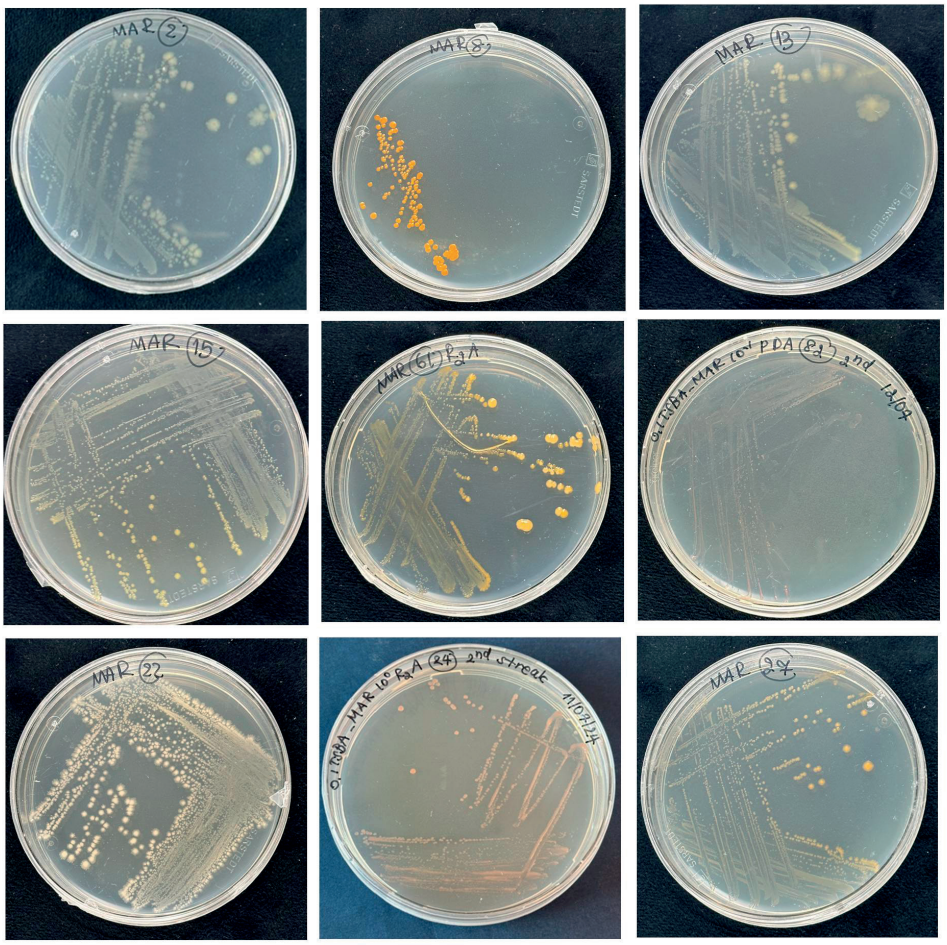
Supplementary Figure 1: Schematic representation of the experimental setup for assessing the effects of bacterial volatiles on Arabidopsis seed germination. (a) Single-plate setup, where one bacterial or buffer control plate was placed in the top-left corner of the germination plate. (b) Double-plate setup, with two bacterial or buffer control plates positioned in the top-left and bottom-right corners to generate a higher volatile concentration. Seeds were exposed only to volatiles, without direct bacterial-seed contact.



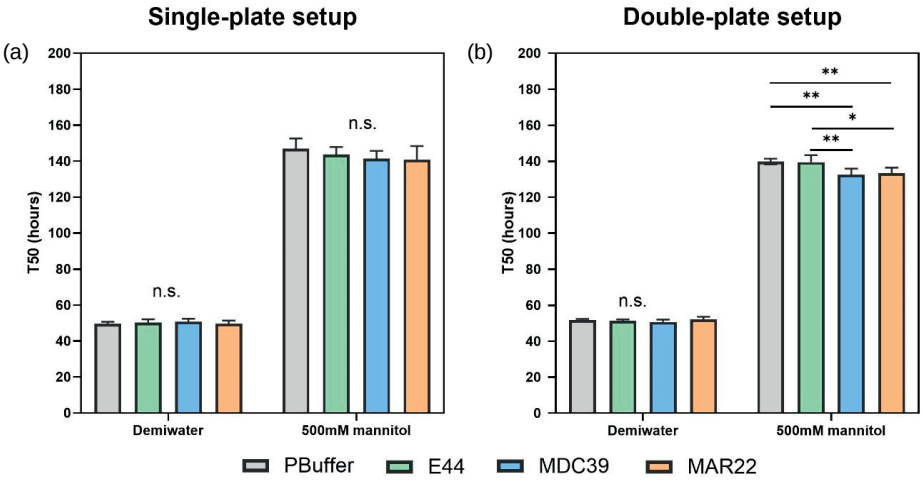
Supplementary Figure 2: Taxonomic profiles comparison of *Arabidopsis thaliana* seed microbiomes between wild and greenhouse populations. Wild populations include MAR, MDC, and POB, while greenhouse populations consist of four accessions (Col-0, L*er*, Bay, and Sha) propagated for three generations on different substrates: rockwool (Rw), commercial potting substrate (Po), gamma-sterilized field soil (St), and non-sterile field soil (Fs). (a) Relative abundance of the ten most dominant bacterial genera (family level shown in parentheses). Genera outside the top ten in overall abundance were grouped under "Other" for visualization clarity. Taxa labelled as "uncultured" were identified only by sequencing and cannot be assigned to formal genus, but are reported at higher taxonomic ranks (e.g., family or class). (b) Alpha diversity is measured using the Observed Diversity Index. Statistical differences were assessed using one-way ANOVA followed by Tukey's post hoc test; $P < 0.05$.



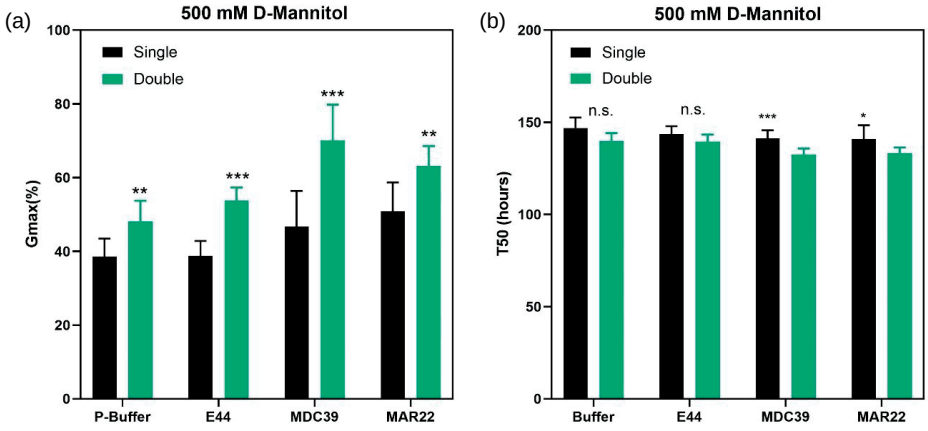
Supplementary Figure 3: Pure bacterial colonies isolated from the MDC population of wild *Arabidopsis thaliana* seeds. Each plate represents an individual bacterial strain identified through 16S rRNA gene sequencing, as listed in Table 1. The figure illustrates the morphological diversity of the cultivable seed endophytes associated with the MDC population.



Supplementary Figure 4: Pure bacterial colonies isolated from the MAR population of wild *Arabidopsis thaliana* seeds. Each plate represents an individual bacterial strain identified through 16S rRNA gene sequencing, as listed in Table 1. The figure illustrates the morphological diversity of the cultivable seed endophytes associated with the MAR population.



Supplementary Figure 5: Effect of bacterial volatiles on *Arabidopsis thaliana* seed germination rate (T50) under non-stress and osmotic stress conditions. Seeds were exposed to volatiles emitted by the seed endophytic strains *Peribacillus frigoritolerans* MDC39 and MAR22, the positive control *Pantoea agglomerans* E44, or phosphate buffer (non-volatile negative control). Germination was assessed under non-stress (demineralized water) and osmotic stress (500 mM D-mannitol) conditions using either a single-plate or double-plate volatile exposure setup. (a-b) Time to reach 50% germination (T50) for single-plate and double-plate setups, respectively. Data represent the mean ± SE; statistical significance was determined by ANOVA followed by Tukey's post hoc test. Significance is indicated as follows: ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.



Supplementary Figure 6: Comparison of germination percentage (Gmax) and germination rate (T50) of *Arabidopsis thaliana* (L*er*) seeds exposed to bacterial volatiles in single- and double-plate setups. Seeds were exposed to volatiles emitted by the seed endophytic strains *Peribacillus frigoritolerans* MDC39 and MAR22, the positive control *Pantoea agglomerans* E44, or phosphate buffer (non-volatile negative control). Germination was evaluated under osmotic stress (500 mM D-mannitol). Panels show the effect of volatile concentration on (a) germination percentage (Gmax) and (b) time to reach 50% germination (T50), comparing single-plate and double-plate volatile exposure. Data represent mean ± SE. Statistical significance was assessed using Independent-Samples t-tests, with the following notation: ns = not significant ($P > 0.05$), * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

SUPPLEMENTARY TABLES

Supplementary Table 1: Collection details for wild *Arabidopsis thaliana* seeds and associated soil samples collected from Spain in 2022. The table includes the date of collection, sampling location, environment where the seeds were collected, and the distance to the target plant from which soil samples were collected (NA indicates that soil samples were not collected).

Date	Population	Seed Sample		Soil sample		Collected location		
		Seed code	Environment where seeds were collected	Soil code	Distance to Target Plant (cm)	Altitude (m)	UTM Easting (ED50)	UTM Northing (ED50)
10-5-2022	Marjaliza	MAR1	On rocks w/ mosses. Surrounded by Cistus & Quercus	MAR_Soil1	20 cm away from plant	993	420277	4381953
		MAR2						
10-5-2022	Marjaliza	MAR3	On soil w/ diverse vegetation. Canopy: Juniperus	MAR_Soil2	5 cm away from plant	1001	420204	4381928
		MAR4						
		MAR5						
		MAR6						
10-5-2022	Marjaliza	MAR7	On soil w/ diverse vegetation. Canopy: Juniperus & Quercus	MAR_Soil3	5 cm away from plant	1002	420198	4381933
		MAR8						
10-5-2022	Marjaliza	MAR9	On rocky soil w/ diverse vegetation. Opening	MAR_Soil4	20 cm away from plant	1033	420072	4381940
		MAR10						
10-5-2022	Marjaliza	MAR11	On rocks w/ mosses. Surrounded by Cistus & Rosmarinus	MAR_Soil5	5 cm away from plant	1023	420138	4381969

Supplementary Table 1: (Continued). Column headers are repeated for clarity.

Date	Population	Seed Sample		Soil sample		Collected location		
		Seed code	Environment where seeds were collected	Soil code	Distance to Target Plant (cm)	Altitude (m)	UTM Easting (ED50)	UTM Northing (ED50)
10-5-2022	Marjaliza	MAR12	On soil w/o vegetation. Canopy: Quercus. Roadside	MAR_Soil6	5 cm away from plant	990	420353	4381900
		MAR13						
10-5-2022	Marjaliza	MAR14	On rocky soil w/ diverse vegetation. Canopy: Quercus. Roadside	MAR_Soil7	5 cm away from plant	990	420357	4381895
		MAR15						
10-5-2022	Marjaliza	MAR16	On rocky soil w/diverse vegetation. Canopy: Quercus. Roadside	MAR_Soil8	5 cm away from plant	985	420369	4381939
11-5-2022	Moral de Calatrava	MDC1	On soil w/ vegetation. Canopy: Quercus	MDC_Soil1	0 cm (Right to the plant)	756	454223	4303426
11-5-2022	Moral de Calatrava	MDC2	On soil w/o vegetation. Canopy: Quercus	MDC_Soil2	5 cm away from plant	756	454168	4303359
		MDC3						
11-5-2022	Moral de Calatrava	MDC4	On soil w/ vegetation. Canopy: Quercus	MDC_Soil3	5 cm away from plant	740	454175	4303348
		MDC5						
11-5-2022	Moral de Calatrava	MDC6	On soil w/o vegetation. Canopy: Quercus	MDC_Soil4	5 cm away from plant	740	454197	4303350
11-5-2022	Moral de Calatrava	MDC7	On soil w/ vegetation. Canopy: Quercus	MDC_Soil5	5 cm away from plant	740	454239	4303276
11-5-2022	Moral de Calatrava	MDC8	On soil w/ vegetation. Canopy: Quercus	MDC_Soil6	0 cm (Right to the plant)	740	454243	4303265

Supplementary Table 1: (Continued). Column headers are repeated for clarity.

Date	Population	Seed Sample		Soil sample		Collected location		
		Seed code	Environment where seeds were collected	Soil code	Distance to Target Plant (cm)	Altitude (m)	UTM Easting (ED50)	UTM Northing (ED50)
		MDC9						
11-5-2022	Moral de Calatrava	MDC10	On soil w/ vegetation. Canopy: Quercus	MDC_Soil7	0 cm (Right to the plant)	733	454158	4303288
11-5-2022	Moral de Calatrava	MDC11	On soil w/o vegetation. Canopy: Quercus	MDC_Soil8	0 cm (Right to the plant)	733	454164	4303252
11-5-2022	Moral de Calatrava	MDC12	On soil w/ vegetation. Canopy: Quercus	MDC_Soil9	5 cm away from plant	728	454147	4303258
		MDC13						
11-5-2022	Moral de Calatrava	MDC14	On soil w/ vegetation. Canopy: Quercus	MDC_Soil10	0 cm (Right to the plant)	728	454183	4303218
		MDC15						
17-5-2022	Poblet	POB1	Opening. Surrounded by Euphorbia, Ulex, Thlaspi, Erysimum	NA	NA	660	334926	4580112
17-5-2022	Poblet	POB2	Understory. Canopy: Quercus, Acer, Rubus	NA	NA	665	334945	4580119
17-5-2022	Poblet	POB3	Opening. Surrounded by Euphorbia, Ulex, Rubus, Dorycnium	NA	NA	664	334989	4580149
17-5-2022	Poblet	POB4	Opening. Surrounded by Euphorbia, Ulex, Rubus, Cistus	NA	NA	662	334989	4580147
17-5-2022	Poblet	POB5	Opening. Surrounded by Cistus, Dorycnium, Erysimum	NA	NA	661	335001	4580150

Supplementary Table 1: (Continued). Column headers are repeated for clarity.

Date	Population	Seed Sample		Soil sample		Collected location		
		Seed code	Environment where seeds were collected	Soil code	Distance to Target Plant (cm)	Altitude (m)	UTM Easting (ED50)	UTM Northing (ED50)
17-5-2022	Poblet	POB6	Understory. Canopy: Arbutus, Quercus, Ruscus	NA	NA	661	335005	4580152
17-5-2022	Poblet	POB7	Opening. Surrounded by Quercus	NA	NA	657	335037	4580171
17-5-2022	Poblet	POB8	Opening. Surrounded by Rubus, Arbutus, Erysimum, Cistus	NA	NA	650	335071	4580206
17-5-2022	Poblet	POB9	Opening. Surrounded by Asparagus, Thlaspi, Geranium	NA	NA	651	335100	4580210
17-5-2022	Poblet	POB10	Opening. Surrounded by Cistus, Prunus	NA	NA	667	334890	4580024
17-5-2022	Poblet	POB11	Opening. Surrounded by Cistus	NA	NA	667	334891	4580026
17-5-2022	Poblet	POB12	Undestory. Canopy: Quercus, Acer, Arbutus, Cistus	NA	NA	672	334916	4580089

Supplementary Table 2 (Excel file): Operational taxonomic units (OTUs) detected in wild and greenhouse *Arabidopsis thaliana* seed samples based on 16S rRNA amplicon sequencing. The table includes OTU IDs, taxonomic assignments at different ranks (from phylum to genus), and their corresponding abundance across all seed samples from the three wild populations (MAR, MDC, POB) and the greenhouse-grown population. The complete dataset is available at: Supplementary Table 2_Chapter 4

Supplementary Table 3: Shared and unique seed endophytic bacterial genera across wild *Arabidopsis thaliana* populations. Endophytic bacterial genera detected in seeds of the three wild populations (MDC, MAR, and POB) are classified as either shared among populations or unique to a single population. Taxa were identified by 16S rRNA gene amplicon sequencing and are reported at five taxonomic ranks: phylum, class, order, family, and genus.

Shared/Unique	Phylum	Class	Order	Family	Genus
MAR_POB	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
MAR_POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
MAR_POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
MAR_POB	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
MAR_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Morganellaceae*	*Buchnera*
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Morganellaceae*	__
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Escherichia-Shigella*
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	__	__
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	__
MAR_MDC	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Marvinbryantia*
POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	__
POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020_group*
POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*[Eubacterium]_xylanophilum_group*
POB	Firmicutes	*Clostridia*	*Clostridia_UCG-014*	*uncultured_bacterium*	__
POB	Firmicutes	*Clostridia*	*Oscillospirales*	*[Eubacterium]_ coprostanoligenes_ group*	*uncultured_bacterium*
POB	Firmicutes	*Clostridia*	*Oscillospirales*	*Butyricicoccaceae*	*UCG-009*

Supplementary Table 3: Continued

Shared/Unique	Phylum	Class	Order	Family	Genus
POB	Firmicutes	*Bacilli*	*Erysipelotrichales*	__	__
POB	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	__
POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	__
POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella_9*
POB	Desulfobacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
POB	Desulfobacterota	__	__	__	__
MAR	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
MAR	Proteobacteria	*Gammaproteobacteria*	__	__	__
MAR	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	__
MAR	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	__	__
MAR	Proteobacteria	*Alphaproteobacteria*	*Acetobacterales*	*Acetobacteraceae*	*Roseomonas*
MAR	Actinobacteriota	*Actinobacteria*	*Micrococcales*	*Cellulomonadaceae*	*Cellulomonas*
MAR	Actinobacteriota	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Clavibacter*
MAR	Actinobacteriota	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*Pseudonocardia*
MAR	Actinobacteriota	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Conexibacter*
MAR	Actinobacteriota	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Solirubrobacter*
MAR	Actinobacteriota	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*
MAR	Acidobacteriota	*Subgroup_22*	*uncultured_bacterium*	__	__
MAR	Acidobacteriota	__	__	__	__
MAR	Firmicutes	*Clostridia*	*Oscillospirales*	__	__
MAR	Firmicutes	*Desulfitobacteriia*	*Desulfitobacteriales*	__	__
MDC	Proteobacteria	*Gammaproteobacteria*	*Diplorickettsiales*	*Diplorickettsiaceae*	*Rickettsiella*
MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Erwiniaceae*	*Pantoea*

Supplementary Tables 4–5 are available at: Supplementary Tables 4–5 Chapter 4 and contains the following data sheets:

Supplementary Table 4 (Excel file): Core and unique bacterial taxa in MAR wild *Arabidopsis thaliana* seeds and surrounding soil. This table lists bacterial taxa that are shared between seeds and bulk soil, as well as those unique to seeds or unique to the surrounding soil. Taxa were identified through 16S rRNA gene amplicon sequencing and are reported across five taxonomic ranks: phylum, class, order, family, and genus.

Supplementary Table 5 (Excel file): Unique bacterial taxa in MDC wild *Arabidopsis thaliana* seeds and surrounding soil. This table lists bacterial taxa that are unique to seeds or unique to the surrounding soil. Taxa were identified through 16S rRNA gene amplicon sequencing and are reported across five taxonomic ranks: phylum, class, order, family, and genus.

Supplementary Table 6: Seed endophytic bacterial genera across wild and greenhouse *Arabidopsis thaliana* populations. This table show the overlap and uniqueness of bacterial genera at the genus level among wild populations (MAR, MDC, POB) and greenhouse populations grouped by substrate type (rockwool, commercial potting substrate, gamma-sterilized field soil, and non-sterile field soil). Taxa were identified by 16S rRNA gene amplicon sequencing and are reported at five taxonomic ranks: phylum, class, order, family, and genus.

Subset	Phylum	Class	Order	Family	Genus
Field	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Tannerellaceae*	*Parabacteroides*
Field	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Rikenellaceae_RC9_gut_group*
MAR	Acidobacteriota	___	___	___	___
MAR	Acidobacteriota	*Subgroup_22*	*uncultured_bacterium*	___	___
MAR	Acidobacteriota	*Actinobacteria*	*Micrococcales*	*Cellulomonadaceae*	*Cellulomonas*
MAR	Acidobacteriota	*Actinobacteria*	*Pseudonocardiales*	*Pseudonocardiaceae*	*Pseudonocardia*
MAR	Acidobacteriota	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Conexibacter*
MAR	Acidobacteriota	*Thermoleophilia*	*Solirubrobacterales*	*Solirubrobacteraceae*	*Solirubrobacter*
MAR	Acidobacteriota	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Clavibacter*
MAR	Acidobacteriota	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Microbacterium*
MAR	Firmicutes	*Desulfitobacteriia*	*Desulfitobacteriales*	___	___
MAR	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
MAR	Proteobacteria	*Gammaproteobacteria*	___	___	___
MAR	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	___
MAR	Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	___	___
MAR	Proteobacteria	*Alphaproteobacteria*	*Acetobacterales*	*Acetobacteraceae*	*Roseomonas*
MDC	Proteobacteria	*Gammaproteobacteria*	*Diplorickettsiales*	*Diplorickettsiaceae*	*Rickettsiella*
MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Erwiniaceae*	*Pantoea*
POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella_9*
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Morganellaceae*	*Buchnera*

Supplementary Table 6: Continued

Subset	Phylum	Class	Order	Family	Genus
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Morganellaceae*	___
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Escherichia-Shigella*
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	___	___
MAR_MDC	Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	___
MAR_POB	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
Sterile_Field	Cyanobacteria	___	___	___	___
Sterile_Field	Firmicutes	*Clostridia*	*Lachnospirales*	___	___
Potting_Field	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Muribaculaceae*	___
Potting_Field	Firmicutes	*Clostridia*	*Clostridia_vadinBB60_group*	___	___
Potting_Field	Firmicutes	___	___	___	___
Rockwool_Field	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	*Colidextribacter*
Rockwool_Field	Firmicutes	*Clostridia*	___	___	___
Rockwool_Field	Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Candidatus_Arthromitus*
Rockwool_POB	Firmicutes	*Clostridia*	*Clostridia_UCG-014*	*uncultured_bacterium*	___
Rockwool_Potting	Deferribacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*uncultured*
Rockwool_Potting_Sterile_Field	Bacteroidota	*Bacteroidia*	*Bacteroidales*	___	___
Rockwool_Potting_Sterile_Field	Deferribacterota	*Deferribacteres*	*Deferribacterales*	___	___
Rockwool_Potting_Sterile_Field	Firmicutes	*Bacilli*	*Lactobacillales*	___	___
Rockwool_Potting_Sterile_Field	Firmicutes	*Clostridia*	*Oscillospirales*	*Oscillospiraceae*	___
Rockwool_Potting_Sterile_Field	Firmicutes	*Bacilli*	*Erysipelotrichales*	*Erysipelotrichaceae*	___
Rockwool_Potting_Sterile_Field_MAR	Firmicutes	*Clostridia*	*Oscillospirales*	___	___

Supplementary Table 6: Continued

Subset	Phylum	Class	Order	Family	Genus
Rockwool_Potting_Sterile_Field_MAR_MDC	Firmicutes	*Bacilli*	*Lactobacillales*	*Carnobacteriaceae*	*Carnobacterium*
Rockwool_Potting_Sterile_Field_MAR_MDC	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_UCG-001*
Rockwool_Potting_Sterile_Field_MAR_POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*
Rockwool_Potting_Sterile_Field_MAR_POB	Bacteroidota	*Bacteroidia*	*Bacteroidales*	*Marinifilaceae*	*Odoribacter*
Rockwool_Potting_Sterile_Field_MAR_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*ASF356*
Rockwool_Potting_Sterile_Field_MAR_POB	Proteobacteria	*Gammaproteobacteria*	*Burkholderiales*	*Sutterellaceae*	*Parasutterella*
Rockwool_Potting_Sterile_Field_POB	Deferribacterota	*Desulfovibrionia*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*
Rockwool_Potting_Sterile_Field_POB	Deferribacterota	___	___	___	___
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Bacilli*	*Erysipelotrichales*	___	___
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	___
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Roseburia*
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	___
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Eubacterium_xylanophilum_group*
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnoclostridium*
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Lachnospiraceae_FCS020_group*
Rockwool_Potting_Sterile_Field_POB	Firmicutes	*Clostridia*	*Oscillospirales*	*Butyricicoccaceae*	*UCG-009*
Rockwool_Sterile_Field	Firmicutes	*Clostridia*	*Clostridia_vadinBB60_group*	*uncultured_bacterium*	___
Rockwool_Sterile_Field_POB	Firmicutes	*Clostridia*	*Lachnospirales*	*Lachnospiraceae*	*Marvinbryantia*
Sterile_Field_POB	Firmicutes	*Clostridia*	*Oscillospirales*	*Eubacterium_coprostanoligenes_group*	*uncultured_bacterium*

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AUTHOR CONTRIBUTIONS

S.S. designed and performed the experiments, analysed the germination data, and wrote the manuscript.

W.P. analysed the microbial amplicon sequencing data, generated the microbial community figures, and contributed to writing the manuscript (Materials and Methods).

C.A.B. and X.P. collected wild *Arabidopsis thaliana* seeds and associated soil samples from field sites in Spain.

P.G. and S.G. assisted with microbial DNA extraction and isolation of seed bacterial endophytes.

M.A.S.A., R.K., and L.B. supervised the project, designed the experiments and edited the manuscript.

Chapter 5

General discussion

Seeds are complex reproductive structures containing an embryo, nutrient-storage tissues (e.g. endosperm), and a protective seed coat that together support survival, dispersal, and establishment of the next generation (Bewley et al., 2013; Sliwinska & Bewley, 2014). In addition, seeds host a diverse community of microorganisms, including seed endophytes, which reside within seed tissues and form a distinct and functionally important component of the seed microbiome (Kumar et al., 2024). Compared with microbial communities in other plant compartments, such as roots and leaves, seed endophytic communities are generally less abundant (Xu et al., 2026); however, they are often more specialized and unique, suggesting that strong selective processes shape their composition (Romão et al., 2025). Despite their relatively low abundance, seed endophytes play an important role in the early stages of the plant life cycle, including seed germination and seedling establishment, especially under stress conditions (War et al., 2023). Seed endophytes can be vertically transmitted from one generation to the next, allowing plants to inherit beneficial microbial traits through seeds (Shahzad et al., 2018). This highlighting their potential role in plant adaptation and stress resilience. One key function of seed endophytes in promoting plant performance under stress is their ability to produce volatile organic compounds (VOCs), such as alcohols, sulfides, ketones, and terpenes (Marzouk et al., 2021). These compounds act as long or short-distance signaling molecules, influencing plant growth and enhancing stress tolerance without direct physical contact (Türksoy et al., 2025). Importantly, the assembly of seed endophytic communities is strongly shaped by both environmental conditions and the genetic background of the host plant, reflecting the combined effects of external factors and host selection on microbial composition and function (Wang & Zhang, 2023). The main objective of this thesis was to investigate how growth environments influence the assembly of seed-associated microbial communities and to evaluate the potential role of seed endophytic bacteria in promoting seed germination and seedling growth under abiotic stress. In this final chapter, I summarize the main findings of this thesis, discuss the remaining unanswered questions, including methodological limitations and aspects that were not fully resolved in the previous chapters, and provide recommendations for future research. In addition, I propose potential strategies to apply seed endophyte knowledge in sustainable agriculture, particularly to improve crop resilience under environmental stress.

SEED MICROBIOMES AS ECOLOGICAL SIGNATURES AND RESERVOIRS OF BENEFICIAL MICROORGANISMS

Seed-associated microorganisms can partially reflect the environmental conditions experienced by the maternal plant (Fort et al., 2021). This is because seeds acquire microbial communities from both the maternal plant tissues and the surrounding environment during seed development and maturation (Abdelfattah et al., 2023). Although environmental conditions are well known to shape plant-associated microbial communities, relatively few studies have examined whether these effects are also reflected on seed microbiomes (Mahmoudi et al., 2024; Morales Moreira et al., 2021). In natural ecosystems, plants are exposed diverse environmental conditions and microbial sources, which may promote recruitment of a wider range of microorganisms into seeds, whereas controlled environments may limit this recruitment (Rodríguez et al., 2020; Wassermann et al., 2019). Consistent with this, greenhouse-grown seed populations examined in Chapter 2 showed lower microbial diversity and fewer unique taxa, together with a greater abundance of human-associated taxa. In contrast, the Mediterranean wild seed populations examined in Chapter 4 contained more diverse and distinct microbial communities, including potentially beneficial microorganisms and taxa associated with natural environmental exposure, such as insect-associated taxa. These contrasting patterns indicate that parental growth environment shapes the seed microbial communities, including taxa associated with specific environmental conditions. This supports the idea that seed microbiomes may act as ecological signatures reflecting the environmental conditions experienced by the maternal plant.

Complex natural environments expose plants to multiple stresses that can shape seed microbial assembly by selectively enriching microorganisms adapted to those conditions (Mao et al., 2024). For example, drought-associated environments have been reported to enrich drought stress-tolerant microbial groups such as Actinobacteria in wheat seed microbiomes, including genera such as *Arthrobacter* and *Curtobacterium*, which were also associated with improved plant growth and drought tolerance (Hone et al., 2021). Similarly, cadmium-exposed plants were likewise shown to selectively enrich cadmium tolerant seed-associated taxa, including *Aeromicrobium* and *Pseudonocardia* (Truyens et al., 2016). These findings suggest that environmental stress can leave detectable signatures in seed microbial communities through the selective recruitment of beneficial microorganisms adapted to the conditions experienced by the parental plant. Moreover, if these beneficial microorganisms are maintained across generations through seed transmission (Abdelfattah et al., 2021), they

could potentially contribute to adaptation to similar environmental stresses in successive generations, although the stability and functional significance of this transmission require further investigation. In contrast, plants grown in less complex environments, such as highly managed agricultural systems, may experience reduced exposure to environmental microbial sources, which could limit recruitment of diverse beneficial microorganisms into progeny seeds (Michl et al., 2024). This could contribute to reduced resilience during seed germination, seedling establishment, and subsequent plant development under more variable environmental conditions. Introducing beneficial seed-associated microorganisms into such systems may help strengthen beneficial plant-microbe interactions and improve crop stress resilience (Cardarelli et al., 2022). Consequently, seed microbial communities may provide indirect information about environmental conditions, ecological interactions, and habitat characteristics experienced during plant growth and seed development, and may also offer opportunities for improving sustainable agricultural practices.

The findings of this thesis further suggest that seeds from plants growing in complex natural environments, particularly wild plant populations, represent important reservoirs of beneficial plant-associated microorganisms. Seed endophytic bacterial isolates obtained from wild cabbage (Chapter 2) and wild Arabidopsis populations (Chapter 4) significantly enhanced Arabidopsis germination and seedling growth under osmotic and salinity stress conditions. These results highlight the potential of wild seed-associated microbial communities as sources of microorganisms associated with plant establishment and stress resilience. Understanding how environmental conditions shape seed microbial communities could help to identify beneficial microorganisms and support the development of microbiome-based strategies to improve crop resilience under changing environmental conditions. However, further research is needed to understand how these microorganisms are passed across generations, how long they can be maintained, and whether they remain effective under field conditions.

CHALLENGES IN SEED ENDOPHYTIC MICROBIOME STUDIES

Seed endophyte microbiome studies are particularly challenging because seeds generally contain a low microbial biomass compared with other plant compartments (Bintarti et al., 2022; Mao et al., 2023). In Chapters 2 and 4, microbial DNA was extracted from surface-sterilized Arabidopsis seeds to target endophytic communities. However, surface sterilization likely further reduced

microbial DNA recovery through removal of epiphytic microorganisms (Nelson, 2018; Sanjenbam & Agashe, 2024). Limited seed material, particularly from wild Mediterranean populations (Chapter 4), further constrained microbial DNA yield. Consequently, low microbial biomass increased susceptibility to contamination from reagents, laboratory environments, human handling, and host-derived plant DNA (Eisenhofer et al., 2019; Salter et al., 2014; Wang et al., 2026). In plant microbiome studies, chloroplast and mitochondrial DNA are frequently co-amplified during 16S rRNA sequencing due to their evolutionary origin from cyanobacteria and alpha-proteobacteria (Carvalho et al., 2015; Raven & Allen, 2003). As a result, host-derived sequences can dominate sequencing datasets and reduce the detection of seed-associated microbes (Hussain et al., 2025; Sonett et al., 2024). In Chapters 2 and 4, universal Peptide Nucleic Acid (PNA) clamps and V5-V7 endophytic targeting primers (799F/1193R) were applied during 16S rRNA amplicon sequencing to reduce amplification of chloroplast and mitochondrial DNA and improve detection of seed endophytic bacteria (Beckers et al., 2016; Lundberg et al., 2013; Shi et al., 2023). However, many plant-derived sequences still had to be removed during bioinformatic analysis, indicating that host DNA suppression was incomplete.

Complementary approaches could improve microbial detection in seed microbiome studies with low microbial biomass and high levels of plant-derived DNA. These may include host DNA depletion strategies, optimized sequencing approaches, and alternative bacterial marker genes such as *gyrB* (Liu et al., 2021). Additional host DNA depletion strategies prior to PCR amplification, including differential and Histodenz density gradient centrifugation, have been shown to reduce plant-derived contamination reads during 16S rRNA sequencing while improving recovery of rare and previously uncultured bacterial taxa (Ikeda et al., 2009; Utturkar et al., 2016). However, these approaches may also cause microbial DNA loss and may bias microbial community composition by enriching certain bacterial taxa, while also requiring relatively large amounts of starting material (Wang et al., 2026).

Improved sequencing approaches may further improve microbial detection and taxonomic resolution in seed microbiome studies. In particular, full-length 16S rRNA sequencing using long-read technologies enables sequencing of the complete ~1500 bp 16S rRNA gene, improving species-level bacterial classification compared with short-read sequencing of partial regions such as V3-V4 or V5-V7 (Johnson et al., 2019). Full-length 16S sequencing can also improve detection of closely related, hard to identify, and potentially novel bacterial taxa (Masuda et al., 2024). However, long-read sequencing approaches remain more expensive and may still be affected by PCR bias and plant-derived DNA contamination (Jeong

et al., 2021; Johnson et al., 2019; Karst et al., 2018). Alternatively, sequencing additional 16S rRNA regions alongside V5-V7 using short-read platforms may improve bacterial classification in seed microbiome studies (Kim et al., 2025). Combining multiple short 16S rRNA regions has been shown to improve taxonomic resolution compared with single-region sequencing (Fuks et al., 2018). In addition, different 16S rRNA regions may vary in their ability to detect plant-associated bacterial taxa (Hrovat et al., 2024). These findings suggest that combining complementary 16S rRNA regions with V5-V7 may improve bacterial detection and taxonomic resolution in seed microbiome studies while remaining more cost-effective and accessible than full-length 16S long-read sequencing (Johnson et al., 2019). However, primer selection would still require careful validation in seed endophyte studies because different primers may vary in plant DNA co-amplification and bacterial detection (Lundberg et al., 2013).

Complementary bacterial marker genes such as *gyrB* have also been proposed to improve bacterial community profiling in seed microbiome studies (Chesneau et al., 2020). In *gyrB* based metabarcoding, bacterial *gyrB* primers are used instead of 16S rRNA primers, potentially reducing chloroplast and mitochondrial co-amplification because *gyrB* targets bacteria-specific genetic regions (Barret et al., 2015; Newberry et al., 2023). In addition, *gyrB* offers higher taxonomic resolution than 16S rRNA, allowing better identification of closely related bacterial taxa (Simonin et al., 2023). Several studies have successfully applied *gyrB* sequencing in seed microbiome research, including *Brassica napus*, bean, and radish-associated bacterial communities (Barret et al., 2015; Rochefort et al., 2019b; Simonin et al., 2023). For example, Chesneau et al. (2020) showed that *gyrB* based metabarcoding recovered 10 to 100 times more bacterial reads than 16S rRNA sequencing in bean and radish seeds due to reduced chloroplast and mitochondrial co-amplification, while also improving species-level identification of dominant seed-associated taxa, including closely related *Pseudomonas fluorescens* subgroup strains. However, *gyrB* sequencing can still be limited by smaller reference databases and primer design limitations that reduce amplification efficiency for some bacterial taxa (Richert et al., 2004; Watanabe et al., 2001). Overall, no single approach fully overcomes the challenges of low microbial biomass and host-derived DNA interference in seed endophyte studies. Combining approaches such as PNA clamps, host DNA depletion, improved 16S rRNA sequencing strategies, and alternative marker gene *gyrB* could improve characterization of seed endophytic bacterial communities.

IMPORTANCE OF SEED DEVELOPMENTAL STAGE IN SEED MICROBIOME CHARACTERIZATION

Because microbial community composition changes substantially as seeds develop from the dry state through imbibition, germination, and seedling establishment, researchers should clearly report the seed developmental stage to enable accurate interpretation and comparison of seed microbiome studies, (Gerna et al., 2024; Yang et al., 2025c). Dry seeds represent a physiologically quiescent stage in which desiccation results in very low water availability and strongly reduced metabolic activity (Nelson et al., 2017). Under these conditions, seed-associated microorganisms remain in a dormant or low-activity states, favoring desiccation-tolerant taxa capable of long-term persistence. As a result, microbial communities detected in dry seeds primarily reflect resident microorganisms adapted to survive until reactivation during imbibition and germination (Gerna et al., 2026). During imbibition, water uptake can shift microorganisms within seed tissues and reactivate previously inactive microbial taxa (Gerna et al., 2024). Following germination, metabolic reactivation and seed exudate release increase nutrient availability within germinating seeds, promoting microbial proliferation and changes in community composition. This process can increase microbial richness and promote the enrichment of specific bacterial groups during germination and early seedling establishment (Torres-Cortés et al., 2018; Yang et al., 2025c). As seedlings continue to develop, microbial communities are further reshaped through selective transmission and tissue-specific colonization (Abdelfattah et al., 2021; Chesneau et al., 2022). Consequently, only a subset of seed-associated microorganisms is successfully transmitted to seedlings (Rochefort et al., 2021), while additional microorganisms are recruited from the surrounding environment, further contributing to microbiome assembly in plants (Mohamed et al., 2024). These stage-dependent changes highlight the importance of clearly stating the seed developmental stage used for microbial characterization when reporting the results.

In Chapters 2 and 4, microbial communities were characterized using dry seeds, which mainly reflect resident seed-associated microorganisms under desiccated conditions (Chandel et al., 2021). These communities represent relatively stable microbial assemblages maintained during seed maturation and storage, including inherited seed-associated microorganisms that persist until reactivation during germination (Dutta et al., 2022). However, analyses limited to a single developmental stage, such as dry seeds, may not capture the dynamic microbial changes that occur during later stages of development. As this thesis focuses on early plant developmental stages, including germination and seedling growth, understanding how these resident microbial communities change during

the transition from dry seeds to seed germination and seedling development is essential for interpreting their contribution to early plant development. Future studies should therefore track microbiome transitions from dry seeds through imbibition, germination, and seedling establishment to clarify how seed-associated microorganisms are transmitted and shape microbiome assembly during early plant development.

CAN SEEDS PERCEIVE MICROBIAL VOLATILE CUES?

Microbial volatile organic compounds (VOCs) are mediators of plant-microbe interactions and have been shown to contribute to plant stress mitigation by modulating physiological and molecular responses under adverse environmental conditions (Fincheira et al., 2021). Previous studies have shown that bacterial VOCs can modulate plant transcriptional networks under salinity stress through transcription factors such as Ethylene Response Factor (ERF) and Dehydration-Responsive Element-Binding (DREB), activating genes involved in osmotic adjustment, ion homeostasis, antioxidant defense, and glutathione metabolism that collectively enhance stress tolerance and maintain cellular homeostasis (He et al., 2024; Kaleh et al., 2025). However, relatively little is known about whether plants can perceive microbial volatile cues during the earliest developmental stages, such as seed imbibition, and whether such perception can contribute to stress adaptation.

Previous physiological studies have shown that VOC exposure during seed imbibition can induce lasting developmental effects. Seeds exposed to VOCs emitted by *Bacillus vallismortis* EXTN-1 for 24 hours during early germination exhibited faster germination and improved plant growth compared with non-exposed controls (Dutta et al., 2025). Although the molecular mechanisms underlying these responses were not investigated, these findings suggest that seeds can perceive and respond to microbial volatile cues during imbibition, with effects that persist beyond the period of VOC exposure.

The findings of Chapter 3 extend these observations by providing molecular evidence that seeds can respond to microbial volatile cues during imbibition under stress conditions. Exposure to VOCs emitted by *Pantoea agglomerans* strain E44 improved seed germination under salinity stress, and this was associated with detectable, although relatively subtle, transcriptional responses in imbibed Arabidopsis seeds. Notably, the identification of rescued genes, in which salt-induced transcriptional responses were partially restored in the presence of E44 VOCs, suggests that exposure to microbial volatiles can alleviate some of the

molecular effects of salinity stress during germination. The observation that these responses occurred in imbibed seeds indicates that microbial volatile perception may occur during seed imbibition, potentially prior to radicle emergence. Together with the physiological evidence reported by Dutta et al. (2025), these findings indicate that microbial volatile signaling can influence plant responses during the earliest stages of development and may contribute to improved stress resilience during germination. However, the broader ecological significance of these responses must be interpreted in the context of natural soil complexity and the experimental constraints of laboratory VOC systems.

ECOLOGICAL RELEVANCE AND LIMITATIONS OF VOC EXPERIMENTAL SYSTEMS

In natural environments, plant seeds generally germinate in soil, where they are exposed to complex and diverse VOC blends released primarily by surrounding soil microbial communities (Ali et al., 2025; Poveda, 2021). Within this soil environment, factors such as pH, nutrient availability, moisture, oxygen availability, and soil structure shape microbial community composition and activity, thereby influencing microbial VOC production, consumption, and movement in soil (Abis et al., 2020; Jiao et al., 2023; Srikamwang et al., 2023). In addition to microbially derived volatiles, plant roots and decomposing organic matter continuously release volatile compounds that contribute to the overall belowground volatile environment (McBride et al., 2020; Voyard et al., 2024). These processes determine VOC distribution and concentration in soil, thereby influencing the volatile cues encountered by germinating seeds (Sharifi et al., 2022). Consequently, VOC exposure under natural soil conditions is likely to differ substantially from that generated in a laboratory experimental setup.

In Chapters 3 and 4, seed germination and seedling growth responses to bacterial VOCs were evaluated using closed laboratory systems. Our experimental design was intentionally selected to provide controlled conditions for investigating the direct effects of VOCs emitted by individual bacterial strains and to enable mechanistic assessment of VOC-mediated responses. However, these systems do not fully reflect natural soil conditions because the experiments were conducted under controlled, sterile conditions using individual bacterial strains rather than microbial consortia, which may not reflect the VOC blends produced through microbial interactions in natural soil environments (Audrain et al., 2015). Therefore, the effects of these beneficial microbes may differ when applied under natural soil, field, or agricultural conditions, where interactions among native microorganisms can influence their establishment and VOC production

(Schulz-Bohm et al., 2017). Furthermore, VOC production is strongly influenced by bacterial growth phase, as different developmental stages are associated with distinct volatile emission profiles (Fitzgerald et al., 2020). In our study, bacterial cultures were incubated for two days prior to VOC exposure, but we did not check if the bacterial growth phase had been reached. In addition, VOC concentrations were not measured directly, which limited confirmation of consistent VOC exposure among replicates. These factors may have contributed to variation in VOC composition and quantity among experimental replicates. These limitations indicate that further testing under more ecologically relevant conditions is needed before applying these findings beyond laboratory systems.

Future studies should include direct measurement of VOC concentrations, characterization of emitted volatile profiles, and standardized examination of bacterial growth phase to improve experimental consistency. In addition, validation under natural soil conditions together with experiments using multi-species microbial communities, would better reflect natural VOC complexity and improve understanding of how these responses translate beyond controlled laboratory systems.

INFLUENCE OF CULTURE MEDIA ON THE SUCCESS OF ISOLATING SEED ENDOPHYTIC MICROBES

The selection of culture media is an important factor influencing the recovery and characterization of seed-associated microorganisms in culture-dependent studies (van Essen et al., 2025). In Chapter 4, commonly used culture media, including TSBA, LB, R2A, and PDA, were used to isolate seed endophytic bacteria from wild Arabidopsis seeds. These media were selected because they support the growth of a broad range of microorganisms and are suitable for routine isolation. However, they may preferentially recover fast-growing and readily culturable taxa, potentially limiting the recovery of taxa with more specific growth requirements (Gerna et al., 2022). Using these media, only 17 distinct seed endophytic bacterial strains were isolated (Chapter 4; Table 3). This relatively low number may reflect the limitations of culture-dependent approaches and suggests that some seed-associated microorganisms may not have been recovered using the culture media used in this study. These difficult-to-culture microorganisms may be adapted to the unique conditions of the seed endosphere, including desiccation, limited nutrient availability, and remain dormant within seeds, making them difficult to isolate using standard culture media (Lobato et al., 2025). For example, Ishida and Furuya (2021) reported that bacterial colonies could not be recovered directly from surface-sterilized *Passiflora edulis* seeds under standard culture conditions

but were detected after seed germination in sterile conditions, suggesting that some seed-associated microorganisms may remain metabolically inactive within seeds and only become culturable when conditions become favorable. As a result, slower-growing or less competitive taxa may not be recovered, leading to an incomplete representation of seed microbial diversity (Khanal et al., 2024). Moreover, some bacterial strains isolated in this study were not detected in the amplicon sequencing dataset from the same seed batches. This difference between culture-dependent and sequencing-based approaches may reflect biases associated with both cultivation and sequencing methods, rather than the true absence of these microorganisms (Khanal et al., 2024; Tkalec et al., 2022). These findings highlight the importance of optimizing cultivation strategies to improve recovery of diverse seed-associated microorganisms.

Future studies should improve cultivation strategies that better mimic the seed internal environment, including low-nutrient media and longer incubation periods. In addition, isolation from germinating seeds may facilitate recovery of seed-associated microorganisms that remain metabolically inactive or suppressed within dormant seeds. The use of seed tissue extracts may further improve cultivation by providing chemical cues and nutrients that more closely resemble the natural seed environment. For example, seed-based media prepared from seed tissues have been shown to improve recovery of unique endophytic taxa (Gerna et al., 2022). Together, these approaches may improve the recovery of additional seed-associated taxa not captured by the standard media used in this study and provide more accurate representation of seed-associated microbial diversity.

FUTURE PERSPECTIVES

Seed-associated microorganisms represent a promising resource for improving crop establishment and stress resilience under changing environmental conditions (Romão et al., 2025). The findings of this thesis (Chapters 3 and 4) demonstrate that seed endophytic bacteria can influence seed germination, early seedling establishment, and plant responses to abiotic stress, highlighting their potential for agricultural applications. Realizing this potential will require strategies that enhance the establishment, persistence, and effectiveness of beneficial microorganisms in agricultural systems. The following sections discuss key future directions, including microbial inoculation technologies, the use of synthetic microbial communities (SynComs), and microbiome-assisted plant breeding.

Among the various approaches available for the application of beneficial seed-associated microorganisms, seed coating and biopriming are widely used strategies for delivering microbial inoculants directly to seeds prior to sowing (Fiodor et al., 2023; Rocha et al., 2019). These techniques facilitate the establishment of beneficial microorganisms on seeds and offer cost-effective and sustainable alternatives to conventional chemical seed treatments (Harsonowati et al., 2026; Patta et al., 2023). Because seed-associated microorganisms are adapted to the conditions within seeds and can colonize plants from the earliest stages of development, they represent promising candidates for seed-based inoculation strategies (Samreen et al., 2021). However, the two approaches differ in their application. Seed coating applies microorganisms as a coating layer on the seed surface and is well suited for large-scale application while maintaining seed handling and storage characteristics (Paravar et al., 2023). In contrast, biopriming combines seed hydration with microbial inoculation, promoting early microbial establishment and activating pre-germination processes, although it may reduce seed longevity and storability (Mitra et al., 2021; Sano & Seo, 2019). Previous studies have demonstrated that microbial seed coatings and biopriming can facilitate successful microbial establishment within plant tissues while improving seed germination, seedling vigor, crop productivity, and stress tolerance, highlighting their potential as effective inoculation strategies (Bhuiyan et al., 2024; Chandrika et al., 2024; Cortés-Rojas et al., 2021). Advances in seed coating technologies, including improved carrier materials, controlled-release formulations, and multilayer coating systems, have the potential to improve the effectiveness of microbial inoculants (Ma, 2019; Singh et al., 2026). Similarly, biopriming effectiveness can be further improved through the use of bio-based carrier materials and the optimization of inoculum preparation, drying, formulation, and storage procedures to enhance microbial survival and seed colonization (Gohari et al., 2026). Together, these approaches can improve microbial survival, persistence, and successful colonization during early plant development (Chai et al., 2022; Köhl et al., 2025). Despite their potential, several challenges remain, including maintaining microbial viability and shelf life during storage and ensuring consistent performance across different crops and environmental conditions (Adeboye et al., 2025). Addressing these challenges will be essential for the successful implementation of seed-based microbial technologies in agriculture.

The development of synthetic microbial communities (SynComs) composed of compatible seed-associated microorganisms offers a promising strategy for improving microbiome-based applications in agriculture (Durán et al., 2025). SynComs design aims to combine microorganisms with complementary traits, functions, and interactions to create stable and effective microbial communities

(Jing et al., 2024). Such communities may enhance inoculant performance by integrating multiple beneficial functions, including plant growth promotion, stress tolerance, nutrient uptake, and disease suppression (Panchal et al., 2026). Moreover, SynComs may better reflect the diversity and complexity of natural seed microbial communities, while functionally complementary microorganisms may increase plant resilience under variable environmental conditions (Arnault et al., 2025). Interestingly, SynComs could be incorporated into seed inoculation approaches, including seed coating and seed biopriming, to promote the establishment of beneficial microbial communities during seed germination and seedling establishment (Simonin et al., 2023; Singh et al., 2023). Recent studies have demonstrated that seed coating with synthetic bacterial consortia can improve seedling growth, enhance plant vigour, and suppress plant diseases more effectively than individual microbial applications (Raza et al., 2024), highlighting the potential of SynComs seed treatments as a promising approach for sustainable agriculture. However, the development of effective SynComs requires careful selection of compatible microbial partners and optimization of community composition, as microbial diversity, functional complementarity, strain identity, and relative abundance can all influence community stability and functionality (Delgado-Baquerizo et al., 2025; Jing et al., 2024; Lin et al., 2024). Developing robust SynComs that remain stable and functional under agricultural conditions will be important for realizing their potential as sustainable microbiome-based tools for crop production.

Beyond microbial inoculation strategies, microbiome-assisted plant breeding represents a longer-term strategy for enhancing the recruitment, maintenance, and transmission of beneficial seed-associated microorganisms across plant generations (Kumar et al., 2026). The shifts in seed microbiome composition observed in this thesis (Chapters 2 and 4) indicate that seed-associated microbial communities are shaped by both host genotype and environmental factors, suggesting that host traits influence microbial assembly and persistence. One potential target for microbiome-assisted plant breeding is root exudation, because plant genetics influences root exudate composition, which in turn shapes microbial recruitment and plant microbiome assembly (Feng et al., 2024). Harnessing plant traits associated with the recruitment, maintenance, and vertical transmission of beneficial seed-associated microorganisms through breeding could support the development of crop varieties that more effectively establish and sustain beneficial microbial communities, thereby promoting plant stress resilience and long-term crop performance under variable environmental conditions (Kumar et al., 2026; Kusstatscher et al., 2021). The core seed microbiome and evidence of microbial transmission identified in this thesis further suggest that maintaining beneficial microorganisms in progeny seeds may be an important target for future

breeding and microbiome engineering approaches. However, the effectiveness of beneficial microorganisms can vary among plant genotypes, which may limit the consistency of microbiome-based approaches across different crop varieties (Zhang et al., 2021). Combining microbiome-assisted plant breeding with SynComs inoculation strategies could provide a promising approach for integrating host traits that support beneficial microorganisms with functionally complementary microbial communities, thereby enhancing the stability and effectiveness of microbiome-based interventions in agriculture.

Despite these opportunities, the successful application of seed-associated microorganisms in agriculture remains challenging because microbial performance is often influenced by plant genotype, environmental conditions, and interactions with native microbial communities (Copeland et al., 2025; Timmusk et al., 2017). Consequently, microorganisms that show beneficial effects under controlled laboratory conditions may not consistently perform under field conditions. Addressing these challenges will require approaches that combine beneficial microorganisms, suitable application methods, compatible plant genotypes, and testing under agricultural conditions. Together, such strategies have the potential to support the development of sustainable microbiome-based technologies that improve crop establishment, stress resilience, and agricultural productivity under changing environmental conditions.

In conclusion, this thesis advances our understanding of seed-associated microbial communities and their potential roles in plant establishment and stress adaptation. The findings demonstrate that seed microbial communities are strongly influenced by environmental conditions and host genotype, highlighting the dynamic nature of microbial assembly within seeds. In addition, wild seed-associated microorganisms were identified as valuable reservoirs of beneficial traits that can enhance seed germination, early seedling establishment, and plant performance under abiotic stress. This work further provides evidence that microbial volatile signaling may influence seed responses during very early developmental stages, supporting a previously underexplored role of seed-associated microorganisms in stress adaptation during germination. At the same time, this thesis highlights important technical and ecological challenges associated with studying low-biomass seed microbiomes, including host-derived DNA interference, cultivation bias, and the difficulty of replicating natural microbial interactions under controlled experimental conditions. Addressing these challenges will be essential for improving our understanding of seed microbiome ecology and its potential applications in agriculture. Overall, by advancing knowledge of the factors shaping seed microbiomes, their transmission across generations, and their contributions to plant establishment and stress

resilience, this thesis provides a foundation for future research and supports the development of microbiome-based approaches for sustainable agriculture under changing environmental conditions.

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Summary

SUMMARY

Seeds represent a critical stage in the plant life cycle, supporting reproduction, dispersal, and the successful establishment of new plants. Beyond their role in plant development, seeds harbour diverse microbial communities that serve as the first microbial inoculum of plants and can influence germination, seedling establishment, and stress adaptation. However, our understanding of how these communities are assembled, transmitted across generations, and contribute to plant performance remains limited. This thesis investigated the influence of environmental conditions and host genotype on seed microbiome composition, explored the role of wild seed-associated microorganisms as reservoirs of beneficial traits, and examined the effects of seed endophyte-derived volatile organic compounds (VOCs) on seed germination, seedling growth, and plant responses to abiotic stress. By integrating microbial community profiling, culture-dependent approaches, germination assays, and transcriptomic analyses, this work provides new insights into the factors shaping seed microbiomes and their potential contributions to plant establishment and stress resilience.

Chapter 1 provided a general introduction to seed germination and seedling establishment, the plant and seed microbiome, microbial volatile organic compounds (VOCs), and plant-microbe interactions. The chapter highlighted factors influencing seed microbiome assembly and transmission, the role of seed-associated microorganisms in early plant development and stress adaptation, and the potential contribution of microbial VOCs to plant responses under abiotic stress. It also presented the thesis objectives and overall experimental framework.

Chapter 2 investigated how growth substrate and host genotype influence seed endophytic bacterial communities and seed germination across three successive generations of *Arabidopsis thaliana* under controlled greenhouse conditions. Plants were grown on substrates differing in microbial diversity, and progeny seeds were evaluated for germination under salinity and osmotic stress conditions. The results showed that seed microbiomes were primarily shaped by a stable, vertically inherited core community, while growth substrate and host genotype contributed to variation in community composition. Beneficial lactic acid bacteria, including members of the genus *Carnobacterium* and the order *Lactobacillales*, were enriched in seeds from non-sterile field soils and were associated with improved germination under stress conditions. Together, these findings demonstrate that seed microbiome assembly is shaped by the interplay between vertical and horizontal microbial transmission, host genotype, and environmental conditions.

Chapter 3 examined the effects of volatile organic compounds (VOCs) produced by *Pantoea agglomerans* E44, a seed endophyte isolated from wild cabbage, on Arabidopsis seed germination and seedling growth under abiotic stress. Exposure to E44 VOCs enhanced germination and early seedling development under salinity and osmotic stress, although the magnitude of these effects varied among genotypes. Transcriptomic analyses of imbibed seeds revealed that salinity was the primary driver of gene expression changes, whereas direct transcriptional responses to E44 VOCs were relatively limited. Notably, E44 VOCs mitigated many salt-induced transcriptional responses, particularly genes associated with ribosome biogenesis, rRNA processing, and protein synthesis, suggesting that microbial volatiles help maintain growth-related cellular processes under stress. In addition, VOC exposure promoted the expression of genes involved in stress adaptation and defence responses. Together, these findings provide molecular evidence that seeds can perceive and respond to microbial volatile cues during imbibition and that microbial volatile signalling can alleviate some of the molecular effects of salinity stress during germination.

Chapter 4 investigated seed-associated bacterial communities in wild *Arabidopsis thaliana* populations collected across Spain and compared them with greenhouse-derived seed microbiomes. The results showed that seed microbiome composition was strongly influenced by the parental growth environment, with wild populations harbouring more diverse and population-specific bacterial communities than greenhouse-grown plants. Despite these differences, a small but conserved core microbiome was maintained across populations. Furthermore, culturable seed endophytes *Peribacillus frigoritolerans*, isolated from wild populations, enhanced Arabidopsis seed germination under osmotic stress through VOC-mediated effects. Together, these findings demonstrate that environmental conditions strongly influence seed microbiome composition, while a small conserved core community is maintained across populations, and highlight wild seeds as valuable reservoirs of beneficial microorganisms.

Chapter 5 concluded this thesis by providing a general discussion of the main findings, their ecological and agricultural implications, and remaining knowledge gaps, methodological limitations, and future research directions. It also highlighted important challenges associated with studying low-biomass seed microbiomes, including host DNA contamination, cultivation bias, and the difficulty of replicating natural microbial interactions under controlled experimental conditions. Potential applications of beneficial seed-associated microorganisms, including seed coating, biopriming, synthetic microbial communities, and microbiome-assisted breeding, were also discussed.

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Beyond work, I was also very lucky to have my **Thai community in Wageningen**. Having all of you around made living far away from home much easier and made Wageningen feel like a second home. I will always remember the Thai food, dinners, parties, trips, board game nights, beach volleyball, and countless laughs we shared. **Jinda (Book)**, you are probably the most extroverted person I have ever met, but also one of the most genuine. Especially during the last year of my PhD, you helped me through so much stress. You encouraged me to exercise and play squash, helped me move to my new room, and always made time for me when I needed you. I am really glad to have you as a good friend, and I wish you lots of success with your own PhD journey! **Polly**, you always have so much energy, and I loved our random chats, gossip, and hanging out together. I especially enjoyed playing beach volleyball with you, although I could never keep up with how sporty you are! And of course, I was always happy when you cooked and shared food with us. Now that we are both in Thailand, we should definitely meet up more often. **Jaae**, we arrived in Wageningen on the same day and somehow also left on the same day. We went through many ups and downs of PhD life together. I especially appreciated our chats when I was tired from the lab or when my experiments did not go as planned. I know your journey has not always been easy, but you are strong, and I know you will make it. I wish you all the best with your PhD and everything ahead! **Golf**, I will always remember our chats at the lunch table in Radix, and especially you helping me build my bed. I definitely could not have done it by myself! **N'Ai**, my little sister, you always made sure we never ran out of good desserts! You are such a kind, cute, and caring person, and I loved having you around. **Jay, Yun, and Nick**, I will definitely miss all the laughs and joking around at J'Jay Pochana. **N'Tae**, I really enjoyed our board game nights. **Fern, Yos, Air, Men, and Boat**, our beach volleyball squad, the games would not have been the same without you guys! **Earth and Maii**, I am glad I got to join you on our memorable trip to the UK. **Max**, I always enjoyed catching up with you at the lunch table in Radix. See you again when you are back at Kasetsart! **Park, P'Oat, Prae, and Cheese**, our Friday dinner squad, those dinners and board game nights were always a great way to end the week. **Fasai, Kaew, PunPun, Ploen, Namtarn, Noey, Ratta, P'Nueng, Ann, Earn, Gun, Mook, Maria, Pang1, Pang2, and Tuck**, I am grateful for all the Thai food, activities, and memories we shared together.

I am also grateful to the **Royal Thai Government** for providing the scholarship that made it possible for me to pursue my PhD in the Netherlands, and to the

Rice Department for giving me the opportunity to continue my studies and supporting me throughout this journey.

Outside my PhD life, I am very grateful to all my friends who stayed close despite the distance. **J'Ning**, even though we were on opposite sides of the world, you never disappeared from my life. You kept calling, checking in, and reminding me that distance did not change our friendship. **Nui and Aommy**, whenever I came back to Thailand for a short visit, I could always count on dinner, a chat, and a good catch-up with you. **Ae**, my best friend, no matter how far away I was, our friendship remained the same. I am so glad that after all these years, we are still close. See you when you are back in Thailand! :) To all **my OKUS29 friends, Ling, Lugyee, Pi, Juang, May, Alex, Ma, Nizzy, SomPi, Kung, and Waew**, I am grateful for a friendship, love, and support that have lasted since our high-school days. A special thank you for also being my bridesmaids and sharing such an important day with me. To all my **MAE16 friends, Mayya, Aof, Vo, Yok, Fon, Cat, Chain, Kai, Nit, P'Oil, Pang, P'Ed, and P'Dong**, I am grateful that no matter where life has taken us, we have always stayed connected. **P'Dong**, you will always be missed by all of us. Rest in peace. To my **Wageningen crew, Ploy, Fai, Pin, N'Porn, P'Good, P'Jae, P'Nong, P'Aum, and P'Ung-Ing**, I am so glad Wageningen brought us together. I hope our little crew will always stay close no matter where we are. **Peach**, I will always remember the delicious food at Obthalay and your kindness to me. Enjoy this new chapter as a mum of two, and I wish you and your family lots of happiness!

Finally, I would like to thank the people closest to my heart, my family, who have been behind me throughout this entire journey. To my husband, **Wasin**, my best friend, my partner, and even my co-author, you have been beside me through everything. You helped me with my work, listened when things were difficult, and shared all the ups and downs of this PhD journey with me. I always knew I could count on you, both in my work and in my life. I am very grateful to have you in my life and for the way you have always believed in me. I love you, and I am so happy that we went through this journey together. This PhD chapter may be coming to an end, but I look forward to everything that is still ahead of us and all the new chapters we will share together. To my **Dad**, your love has always shown in the things you do for me. Whenever I need you or simply call, you are there without hesitation. I know I can always count on you. I love you, Dad. To my little sister, **Mo**, we have been through a lot together, and even though we are now far apart, you will always be my little sister no matter where we are. Good luck with your new journey in the US. I hope we will see each other again very soon! And **P'Tum**, I am grateful that you are there for Mo and take such good care of my little sister. To my parents-in-law, **Mae Tuk and Por Dang**, I am very

grateful for the way you have welcomed me and loved me like one of your own. I feel very lucky to have you both as part of my family. To **Nar Kob, Nar Morn, Nar Mod, Khun Berge, and Bam**, having you here on my PhD defence day means a lot to me, and I am happy to share this special moment with you.

And finally, to my **Mum**, I love you and miss you every day. You are the person who made me who I am today. You gave me a good education and always wanted me to continue studying and go as far as I could. And Mum, I did it. I finally made it here. Even though you are no longer here with me to see this day, I know you would be happy and proud of me. You will always be in my heart and always be a part of who I am. This PhD is for you, Mum. I love you, and I will carry you with me forever.

About the author

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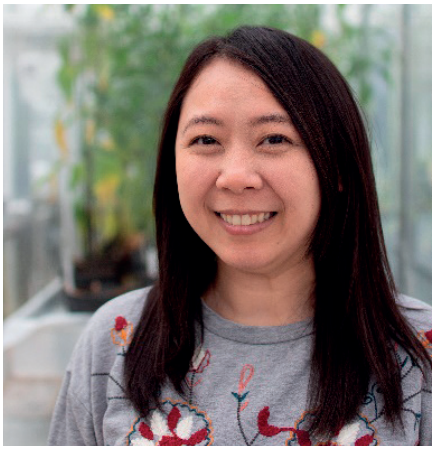

Sasiwimon Siricharoen was born on September 17th, 1986, in Bangkok, Thailand. Her interest in plants and nature began during her high-school years, when she took courses in agriculture and vegetable production. She especially enjoyed working with plants, and this early interest inspired her to continue her studies in agricultural science. In 2007, she obtained her bachelor's degree in Entomology from Kasetsart University.

After graduating, Sasiwimon started working at the Rice Department, Ministry of Agriculture and Cooperatives, Thailand, as an Agricultural Research Officer in the Quality Control Group, Rice Seed Division. Working with rice seed quality brought her closer to the practical side of seed science and gradually strengthened her interest in seed quality and seed physiology.

In 2014, she obtained a Master of Science in Agricultural and Resource Economics from Kasetsart University, broadening her perspective on agriculture. In the same year, she received a Royal Thai Government scholarship to pursue a second master's degree in Plant Sciences at Wageningen University & Research, the Netherlands, specializing in Greenhouse Horticulture. During her studies at Wageningen, her research focused on seed and seedling performance in tomato and rice, further developing her interest in seed science. After completing her degree in 2017, she returned to Thailand and continued her work at the Rice Department.

In 2021, Sasiwimon received another Royal Thai Government scholarship to return to Wageningen University & Research, this time to pursue a PhD at the Laboratory of Plant Physiology under the supervision of Prof. dr. Leónie Bentsink, dr. Rumyana Karlova, and dr. Mariana Silva Artur. Her PhD brought her into the new and fascinating world of the seed microbiome, where she investigated the diversity of seed-associated microorganisms and their potential roles in early plant development and stress resilience.

This doctoral research resulted in the thesis *Hidden allies: Seed microbiome diversity and its potential in early plant development and stress resilience*. After completing her

PhD research in 2026, Sasiwimon returned to Thailand and resumed her position at the Rice Department. She hopes to continue exploring the world of seeds and plants and to use the knowledge and experience she has gained to contribute to seed science and agriculture in Thailand.

Education statements

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Sasiwimon Siricharoen
Date: 09 November 2026
Group: Laboratory of Plant Physiology
University: Wageningen University & Research

1) Start-Up Phase	date	credits
► **First presentation of your project**		
Plant Physiology (PPH) seminar	7 April 2022	1,5
► **Writing or rewriting a project proposal**		
Writing proposal project	1 October 2021	6,0
► **MSc courses**		
Subtotal Start-Up Phase		7,5

2) Scientific Exposure	date	credits
► **EPS PhD days**		
EPS PhD days - Get2Gether, Soest, NL	3 and 4 May 2022	0,6
EPS PhD days - Get2Gether, Soest, NL	1 and 2 May 2023	0,6
► **EPS theme symposia**		
EPS Theme 3 – Metabolism and Adaptation, Utrecht, NL	7 November 2023	0,3
EPS Theme 3 – Metabolism and Adaptation, Wageningen, NL	4 October 2024	0,3
► **National platform meetings**		
Annual Experimental Plant Sciences Meeting, Lunteren, NL	11 and 12 April 2022	0,6
Annual Experimental Plant Sciences Meeting, Lunteren, NL	17 and 18 April 2023	0,6
Annual Experimental Plant Sciences Meeting, Egmond aan Zee, NL	14 and 15 April 2025	0,6
11th Dutch Seed Symposium , Wageningen, NL	3 October 2023	0,3
12th Dutch Seed Symposium, Wageningen, NL	2 October 2024	0,3
► **Seminars (series), workshops and symposia**		
15th Plant-Insect Interaction Workshop	21 October 2021	0,3
MiCRop series by Dr.Stephane Hacquard : Synthetic Ecology of the Root Microbiota: Linking Genes to Communities and Ecosystems	12 September 2022	0,1
MiCRop series by Prof. dr. Eva Stukenbrock : Fungal interactions and co-adaptation in the plant phyllosphere	16 November 2023	0,1
MiCRop series by Dr. Derek S. Lundberg : Sphingomonas, an encyclopedia of genetic secrets to keep bacterial inoculants from dying in the field	16 May 2024	0,1
MiCRop series by Dr. Ashley Shade : Exploring the seed microbiome and its consequences for plant resilience	15 May 2025	0,1
PPH seminar series : Lucio Conti : Regulatory networks linking flowering time and drought adaptation	16 April 2026	0,1
PPH seminar series : Szymon Swiezewski : Molecular Biology of Seed Dormancy	13 Febuary 2026	0,1
► **Seminar plus**		
► **International symposia and congresses**		
5th Plant Microbiome Symposium, Amsterdam, NL	17-21 June 2024	1,2
International miCROPe Symposium 2024, Vienna, AT	15-18 July 2024	1,2
15th Biennial International Society of Seed Science (ISSS) Conference 2025, Perth, AU	15-19 September 2025	1,5
SeedCon2026- International Conference on Seed Microbiomes, Potsdam, DE	5-7 May 2026	0,9
► **Presentations**		
Poster presentation at Annual Experimental Plant Sciences Meeting 2023, Lunteren, NL	17 and 18 April 2023	1,0
Oral presentation at 11th Dutch Seed Symposium, Wageningen, NL	3 October 2023	1,0
Poster presentation at International miCROPe Symposium 2024, Vienna, AT	16 July 2024	1,0
Oral presentation at ISSS (International Society of Seed Science) 2025, Perth, AU	15-19 September 2025	1,0
Poster presentation at SeedCon2026, Potsdam, DE	5-7 May 2026	1,0
Oral presentation at Annual Experimental Plant Sciences Meeting 2025, Egmond aan Zee, NL	14 April 2025	1,0
► **Interviews**		
3rd year interview with an EPS research theme coordinator	24 June 2024	0,7
► **Excursions**		
Excursion to Bayer	22 January 2026	0,2
Subtotal Scientific Exposure		16,8

3) In-Depth Studies	date	credits
► **Advanced scientific courses & workshops**		
PE&RC course Microbial Ecology course, Nunspeet, NL	19-24 June 2022	1,5
17th International Master Class Seed Technology, Wageningen, NL	9-13 October 2022	1,5
► **Journal club**		
PPH literature discussion	2021-2024	1,5
Microbiome literature discussion	2021-2026	1,5
► **Individual research training**		
Subtotal In-Depth Studies		6,0

4) Personal Development	date	credits
► **General skill training courses**		
EPS Introduction Course, Wageningen, NL	1 November 2021	0,3
WGS course Project & Time Management, online	28 April - 9 June 2022	1,5
PE&RC course Basic Statistics, Wageningen, NL	16-24 May 2023	1,5
WGS course Scientific Writing, Wageningen, NL	25 April - 27 June 2024	1,8
WGS course Adobe Illustrator - Scientific Artwork & Infographics, online	3-24 September 2024	1,2
► **Organisation of scientific meetings, PhD courses or outreach activities**		
► **Membership of EPS PhD Council**		
Subtotal Personal Development		6,3

CONTINUED ON NEXT PAGE

5) Teaching & Supervision Duties	*date*	*credits*
► **Courses**		
PPH-31306 Seed Science and Technology	2023-2025	1,0
PPH-10806 Structure and Function of Plants	2023-2024	2,0
► **Supervision of BSc/MSc projects**		
MSc Thesis supervision	November 2022 - May 2023	1,5
MSc Research Practice supervision	January 2024 - May 2024	1,5
Subtotal Teaching & Supervision Duties		6,0

TOTAL NUMBER OF CREDIT POINTS*	**42,6**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Education Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

This work was carried out at the laboratory of Plant physiology, Wageningen University. The research described in this thesis was financially supported by Royal Thai Government scholarship. Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Sasiwimon Siricharoen

Cover image: The image depicts the journey from seed to seedling, highlighting the hidden microbial communities associated with seeds. The microorganisms within and around the seed represent the diverse seed microbiome and its presence throughout germination and early plant development.

Layout by Sasiwimon Siricharoen and ProefschriftMaken.nl

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