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The role of the seed microbiome of barley (*Hordeum vulgare*) as driver for the composition of root endophytes

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Zusammenfassung

Endophyten sind Mikroorganismen, welche das innere Gewebe einer Pflanze besiedeln. Sie sind ubiquitär mit ihnen verbunden und spielen eine wichtige Rolle für die Gesundheit der Pflanzen. Die Samen der Pflanze liefern, wie kein anderes pflanzliches Organ, Einblicke in die Herkunft des pflanzlichen Mikrobioms. Ihr Einfluss auf die Zusammensetzung des pflanzlichen Mikrobioms ist jedoch nach wie vor schwer bestimmbar. Die Struktur und Funktion von Endophyten, die durch das Saatgut weiter gegeben werden, muss jedoch noch charakterisiert werden. In dieser Arbeit wurde Gerste (*Hordeum vulgare*) als Modellpflanze verwendet, um samengetragene Endophyten und ihren Einfluss auf das Wurzelmikrobiom zu untersuchen. Dementsprechend wurden drei Studien, wie im Folgenden beschrieben, durchgeführt.

Die erste Studie charakterisierte Endophyten aus Gerstensamen. Wir untersuchten welche Rolle Endophyten aus Gerstensamen als Quelle für das „erste Inokulum“ für das Wurzelmikrobiom spielen. Wir fanden heraus, dass die Struktur von samenbürtigen Endophyten kulturabhängig ist. Die meisten bakteriellen Endophyten wurden jedoch in allen Samen der verschiedenen Sorten gefunden. Diese bestanden hauptsächlich aus den Gattungen *Phyllobacterium*, *Enterobacter*, *Paenibacillus* und *Pseudomonas*. Unter sterilen Bedingungen ähnelten die Hauptgruppen in Gerstenwurzeln dem Profil des Samenmikrobioms, was auf die vertikale Übertragung von Endophyten von Samen zu Wurzeln hinweist. Samenbürtige Endophyten wurden hingegen weniger häufig im Wurzelmikrobiom gefunden, wenn die Pflanzen in Boden gezüchtet wurden. Stattdessen wurden Actinomycetales, Rhizobiales und Burkholderiales in Gerstenwurzeln angereichert, was auf einen starken Einfluss der Bodenbakteriengemeinschaft hinweist.

Die zweite Studie untersuchte die genomischen Eigenschaften von samenbürtigen Endophyten, die vertikal auf die Wurzeln übertragen werden. Die am häufigsten vorkommenden Funktionen umfassten Chemotaxis, Biofilmbildung und Adhäsion, wie z.B. Methyl-akzeptierende Chemotaxis, Flagellen, Pili und Hämagglutinin. Weitere dominante funktionale Eigenschaften waren die Anwesenheit von Osmoprotektiva und antioxidativen Enzymen, welche entscheidend sind für das Überleben bei hohem osmotischen Druck. Außerdem fanden wir die Enzyme Amylase, Aminopeptidase und Phytase, welche für die Nährstoffmobilisierung während der Samenkeimung entscheidend sind. Zudem waren ACC-

Desaminase- und IAA-Produktion in samengetragenen Endophyten weit verbreitet. Das am häufigsten vorkommende Sekretionssystem war das Typ VI-Sekretionssystem (T6SS). Ebenfalls weit verbreitet war T3SS. Jedoch fehlte die Nadelstruktur des T3SS, wodurch der Begriff des „entwaffneten Erregers“ geprägt wurde.

Das Bodenmikrobiom verleiht den Pflanzen neben dem anfänglichen mikrobiellen Inokulum, das durch Samen bereitgestellt wird, eine extrem hohe Vielfalt. Unsere dritte Studie untersuchte die Auswirkung des Bodenmikrobioms auf die Gersten-Trockenheitstoleranz und die Reaktion von Endophyten auf Trockenstress. Unter gut bewässerten Bedingungen zeigte der Vergleich der Pflanzenaufzucht in natürlichem gegen autoklavierten Boden eine ähnliche Leistung der Gerstenpflanze. Unter Trockenstress zeigten die Pflanzen jedoch eine bessere Leistung in Gegenwart des natürlichen Bodenmikrobioms verglichen mit dem des autoklavierten Bodens mit einem gestörten Mikrobiom. Die Gattung *Massilia* war nach Trockenheit in den Gerstenwurzelendophyten angereichert. OTUs von Actinobacteriales, Rhizobiales und Burkholderiales waren ebenfalls angereichert. In Bezug auf Pilze, waren OTUs, zugeordnet zu *Pleurophragmium* und *Falciphora,* unter Trockenstress in den Wurzeln angereichert. In autoklaviertem Boden konnten vier OTUs in den Wurzeln nachgewiesen werden, die aus den Samen stammen. Diese wurden zugeordnet zu den Gattungen *Pantoea* und *Erwinia*. Zudem wurden viele dürrebedingte Gattungen auch in Gerstensamen nachgewiesen, wenngleich nicht dieselben OTUs. Unsere Ergebnisse deuten auf eine mögliche Korrelation zwischen den von Samen übertragenen Endophyten und dem Wurzelmikrobiom von dürre-gestressten Pflanzen hin.

Zusammenfassend charakterisierten wir die Zusammensetzung und Funktion von Endophyten aus Gerstensamen. Unsere Arbeit beleuchtet die Rolle von samenbürtigen Endophyten als Treiber beim Aufbau von Wurzelmikrobiomen. Auch wenn ihre genaue Rolle für das Pflanzenwachstum noch geklärt werden muss, ist es möglich, dass sie der Pflanzenentwicklung während der Keimung und Etablierung der Sämlinge zugute kommen. Ebenfalls konnte die potenzielle Bedeutung von samenbürtigen Endophyten in Verbindung gebracht werden mit der Toleranz der Wirtspflanze gegenüber Trockenheit.

Abstract

Endophytes are microorganisms colonizing plant internal tissues. They are ubiquitously associated with plants and play important roles in plant health. Seeds, like no other plant organs, provide insights into the origin of plant microbiota. However, their impact on plant microbiota assembly remains elusive. The composition and function of seed-borne endophytes have yet to be characterized. In this thesis, we used barley (*Hordeum vulgare*) as the model plant to investigate seed-borne endophytes and their influence on the root microbiome. Accordingly, three studies were conducted as described below.

The first study characterized barley seed-borne endophytes and investigated the role of seed-borne endophytes as a source of "first inoculum" of root microbiome. We found that the structure of barley seed-borne endophytes was cultivar-dependent. However, most of the bacterial endophytes were shared by seeds of different cultivars, mainly including *Phyllobacterium*, *Enterobacter*, *Paenibacillus*, and *Pseudomonas*. Under sterile conditions, the major groups in barley roots were similar to the profile of seed microbiome, indicating the vertical transmission of endophytes from seeds to roots. When plants were grown in soil, seed-borne endophytes became less abundant in root microbiome. Instead, Actinomycetales, Rhizobiales, and Burkholderiales were enriched in barley roots, indicating a strong influence of the soil bacterial community.

The second study explored the genomic features of the seed-borne endophytes vertically transmitted to roots. The most prevalent functions include chemotaxis, biofilm formation and adhesion, such as methyl-accepting chemotaxis, flagella, pili, and hemagglutinin. Other dominant functional pathways were found to be osmoprotectants and antioxidant enzymes pivotal to survive the high osmotic pressure as well as amylase, aminopeptidase, and phytase, which are critical for nutrient mobilization during seed germination. ACC deaminase and IAA production were widely distributed in seed-borne endophytes. The most abundant secretion system was found to be the Type VI secretion system (T6SS). T3SS was also widely occurred. However, the needle part of the T3SS was missing, coining the term "disarmed pathogen".

Besides the initial microbial inoculum provided by seeds, soil microbiome confers an extremely high diversity to plants. Our third study investigated the effect of soil microbiome

on barley drought tolerance and the response of endophytes under drought stress. We found similar barley plant performance in the natural and autoclaved soil under well-irrigated conditions. However, plants did perform better under drought stress in the presence of the natural soil microbiome compared to autoclaved soil with a disturbed microbiome. Following exposure to drought, *Massilia* was enriched in barley root endophytes. OTUs belonged to Actinobacteriales, Rhizobiales and Burkholderiales were also enriched. With regard to fungi, OTUs assigned to *Pleurophragmium* and *Falciphora* were enriched in roots under drought stress. In the autoclaved soil, 4 drought-enriched root OTUs assigned to *Pantoea* and *Erwinia* were found with seed origin. Besides, many drought-related genera were also detected in barley seeds, although not the same OTUs. Our results indicate a possible correlation between the seed-borne endophytes and the root microbiome of drought-stressed plants.

In summary, we characterized the composition and function of barley seed-borne endophytes. Our work shed light on the role of seed-borne endophytes as drivers in the assembly of root microbiome. Although their exact roles on plant growth still need to be addressed, it is possible that they benefit plant development during germination and establishment of the seedlings. The potential significance of seed-borne endophytes on plant drought tolerance was also implicated.

1. Introduction

1.1 Plant endophytes

Plants host a diverse community of microbes, including bacteria, fungi, archaea, protozoan, and viruses, collectively termed the "plant microbiota" (Schlaeppi and Bulgarelli 2015). Decades of research has demonstrated the importance of microorganism for plant health (Bulgarelli et al. 2013; Berg et al. 2014), such as increased nutrient acquisition (van der Heijden et al. 2016), disease suppression (Mendes et al. 2011; Ritpitakphong et al. 2016), priming of the plant immune system (Van der Ent et al. 2009), induction of systemic resistance (Zamioudis et al. 2015) and increased tolerance to abiotic stresses (Rolli et al. 2015). Recently, the emerging concept of holobiont considers the host plant and the associated microbiota as a functional entity and a unit of selection in evolution (Zilber-Rosenberg and Rosenberg 2008; Vandenkoornhuyse et al. 2015) (Figure 1-1). The plant microbiota are viewed as a reservoir of additional genes to facilitate the adaptation of plants to the constantly changing environment (Rosenberg and Zilber-Rosenberg 2018).

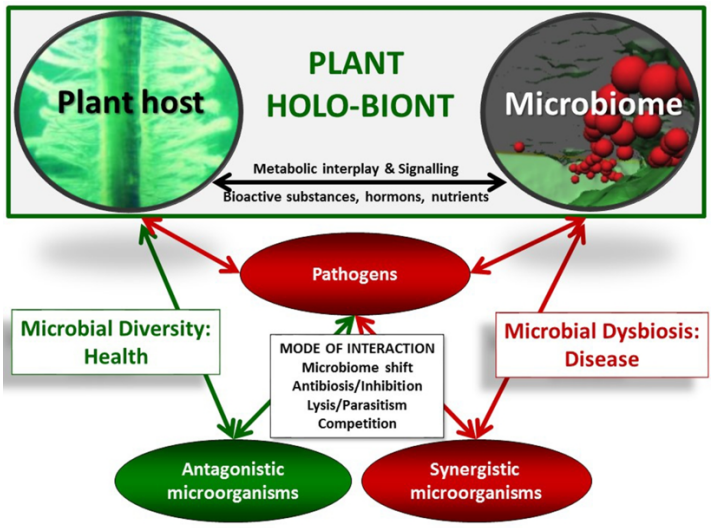


Figure 1-1The illustration of the plant holobiont and related key interactions. Healthy plants are associated with their microorganisms by metabolic cooperation and exchange of signals, hormones and nutrients. Diseases are characterized by a microbial dysbiosis and a response of specific microbes, which can act as antagonists or synergists towards pathogens (Berg et al. 2017).

In nature, microbial consortia form both on and inside healthy and asymptomatic plants (Berg et al. 2016). Microorganisms that colonize plant internal tissues for all or part of their lifetime are termed endophytes (Wilson 1995). They are generally considered to be non-pathogenic, causing no visible disease symptoms to host plant (Hallmann et al. 1997). However, within an apparently healthy plant, the endophytic community could consist of a mix of mutualistic,

commensal, and latent pathogenic strains (Fesel and Zuccaro 2016). The outcome of the plant and microbe association usually depends on environmental circumstances and/or host genotype (Monteiro et al. 2012; Kloepper et al. 2013). Thus, the conceptual aspect of the nature of endophytes is under dispute (Brader et al. 2017). Therefore, Hardoim *et al.* (2015) proposed recently that the term "endophyte" should refer to habitat only.

Endophytes could be classified as 'obligate' or 'facultative' in accordance with their life strategies and intimacy degree with plants (Hardoim et al. 2008). Obligate endophytes are strictly bound to plants and require plant tissues to complete their life cycle, whereas facultative endophytes can grow outside host plants (Steenhoudt and Vanderleyden 2000). Virtually all the plants studied to date were found inhabited by endophytes (Ryan et al. 2008). With the emergence of next-generation sequencing, cultivation-independent analyses have provided deep insights into the community composition of various host plants, such as the widely used model plant *Arabidopsis thaliana* (Bulgarelli et al. 2012; Lundberg et al. 2012), rice (Edwards et al. 2015), maize (Peiffer et al. 2013), lettuce (Rastogi et al. 2012; Cardinale et al. 2015), grapevine (Morgan et al. 2017) and poplus (Cregger et al. 2018). They have all conclusively demonstrated that the plant endosphere is dominated by a few bacterial phyla, mainly Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. In a thorough survey of endophytic data sets, more than 200 genera from 21 phyla of bacterial species have been reported (Hardoim et al. 2015).

Plants constitute vast and diverse niches for endophytic organisms. Endophytic bacteria mostly occupy intercellular spaces in the plant (Kandel et al. 2017). Recently, intracellular colonization of plants by bacteria has also been reported. Bacteria were observed inside plant cells of shoot tip and shoot meristem (Pirttila et al. 2000; Thomas and Reddy 2013; White et al. 2014). Bacterial endophytes have been detected in all plant compartments, including roots, stems, leaves, seeds, fruits, tubers and ovules (Rosenblueth and Martinez-Romero 2006) (Figure 1-2).

The extensive overlap between root and leaf associated community has been described, and the reciprocal relocation between root-associated and leaf-associated bacterial communities has been further validated using microbiota reconstitution experiments with germ-free *A. thaliana* (Bai et al. 2015). Despite of the striking similarities observed between *A. thaliana* leaf- and root- associated bacterial communities, large-scale genome sequencing and re-colonization of germ-free plants revealed that host-associated microbiota members are specialized and adapted to their respective cognate plant organs (Bai et al. 2015). In general,

roots have higher numbers of endophytes compared with above-ground tissues (Turner et al. 2013).

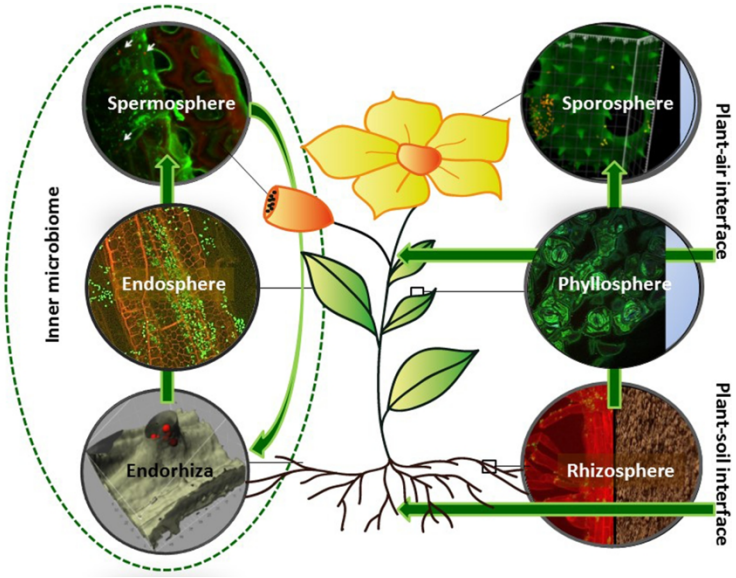


Figure 1-2 The microhabitat of endophytes in different plant compartments (Berg et al. 2017)

Living inside plants, endophytes are closely associated with plants and play crucial roles in plant development, growth, fitness, and diversification (Berg et al. 2014). Endophytes can promote plant growth and confer plants tolerance to biotic and abiotic stresses (Finkel et al. 2017). In brief, endophytes are both ecologically interesting and important in agricultural applications.

1.2 Bacterial seed endophytes

Seeds host diverse microbial communities. The presence of bacteria inside seeds has been visualized by microscopic analyzes coupled with fluorescence in situ hybridization (FISH) (Compant et al. 2011; Cope-Selby et al. 2017; Alibrandi et al. 2018; Glassner et al. 2018). Bacteria were detected in various parts of seeds, including seed coat, cortex, endosperm, embryo, and embryonic hypocotyl-root axis tissues (Cankar et al. 2005; Puente et al. 2009; Cope-Selby et al. 2017). Moreover, different bacterial taxa seem to colonize different niches inside seeds. Recently, taxa-specific colonization patterns were shown in studies of melon seed endophytes (Figure 1-3). Similar results were demonstrated in the seeds of a bee-pollinated legume tree Curupaú. (Cope-Selby et al. 2017; Alibrandi et al. 2018).

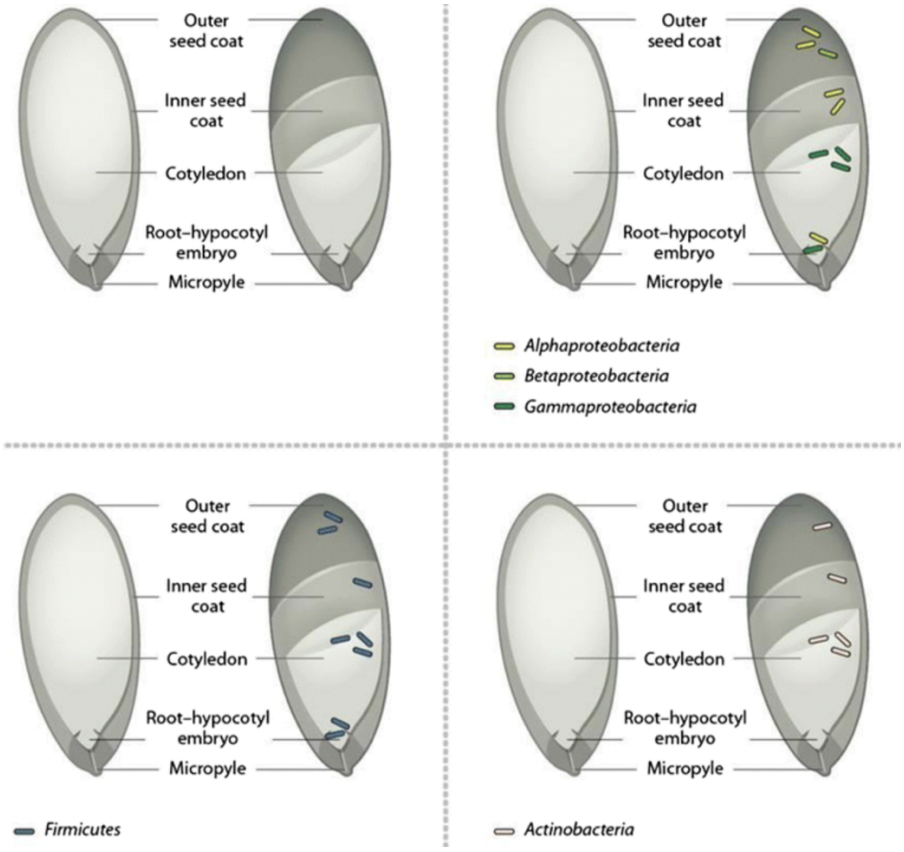


Figure 1-3 The drawing summarizing niches of endophytic bacteria inside seeds of cultivated melon *Cucumis melo reticulatus* group 'Dulce' (Glassner et al. 2018)

Bacterial communities inhabiting seed endosphere are dynamic throughout the process of seed maturation (Okunishi et al. 2005). Culturable bacteria were found to be more diverse in the early stage of seed development than in the middle and late stages (Liu et al. 2013). The community composition also shifts during seed development (Mano et al. 2006).

Seed endophytes may derive from a plethora of sources. Parts of seed microbiota are considered to originate from the soil environment, as many taxa detected within seeds are highly similar to common soil strains. Some bacteria could colonize the root system and spread inside plants via xylem vessels (Nelson 2018). The translocation of bacteria towards reproductive organs was demonstrated in grapevine, where fluorescence-labeled *Burkholderia* strain was traced from the rhizosphere to young berries (Compant et al. 2008). In this manner, the migration of bacteria from soil to seeds is likely to occur. Seed endophytes may also derive from the anthosphere or the carposphere environments (Compant et al. 2010; Compant et al. 2011). A recent study demonstrated the transmission of bacteria from flowers to seeds by inoculating the bacterial strain *Paraburkholderia phytofirmans* PsJN to the flowers of parent plants (Mitter et al. 2017).

Seed endophytes have been studied in many plants such as maize, rice, grapevine (Compant et al. 2011), tobacco (Mastretta et al. 2009), eucalyptus (Ferreira et al. 2008), rapeseed (Graner et al. 2003), coffee (Vega et al. 2005), ash (Donnarumma et al. 2011), soybean (Oehrle et al. 2000), sugarbeet (Dent et al. 2004), pumpkin (Furnkranz et al. 2012), peanut (Sobolev et al. 2013), bean (Rosenblueth et al. 2012), tomato (Xu et al. 2014), *A.thaliana* (Truyens et al. 2013) and various grasses and weeds. The frequently reported bacteria inhabiting seed endosphere include *Bacillus*, *Pantoea*, *Enterobacter*, *Paenibacillus*, *Pseudomonas*, *Staphylococcus*, and *Stenotrophomonas* (Truyens et al. 2015).

The low density and diversity of bacteria in seeds lead to the assumption that only bacterial strains with specific characteristics are able to colonize and survive in seeds. Studies have found that seed-borne endophytes are mostly motile (Okunishi et al. 2005). They are tolerant of high osmotic pressure, as the seed maturation involves starch accumulation and water loss. Bacteria inside seeds often possess amylase in order to utilize starch (Pitzschke 2016). Phytase activity is reported in some endophytes to make use of phytate, the main storage form of phosphorus in seeds (Lopez-Lopez et al. 2010). Endospore formation is another important characteristic of seed colonizers. Spores and other dense structures were observed in Miscanthus seeds, which may protect the seed endophytes and contribute to their long-term survival (Cope-Selby et al. 2017). Other plant growth promotion potentials were also found, such as ACC deaminase, phytohormone production and antifungal activities (Ji et al. 2014; Herrera et al. 2016; Shahzad et al. 2016; Bodhankar et al. 2017; Walitang et al. 2017; Khalaf and Raizada 2018; Khalaf and Raizada 2018).

The composition of seed microbiota can have a direct impact on seed quality (Shade et al. 2017). Seed-borne endophytes contribute to seed preservation (Shahzad et al. 2018), seed dormancy release through the production of cytokinins (Goggin et al. 2015), increased germination rate (Rout et al. 2013) and reduced cadmium phytotoxicity (Mastretta et al. 2009). Seed bacterial endophytes are involved in the establishment of giant cardon cactus on barren rocks. Cactus seeds disinfected with antibiotics halt seedling development. Plant growth was restored by inoculation of the same endophytes that contribute to rock weathering (Puente et al. 2009; Puente et al. 2009). Similarly, the removal of rice seed endophytes by surface-sterilization and antibiotic treatments restricted seedling growth and development (Verma et al. 2017)

Seed endophytes are of particular interest, as they may be transmitted from generation to generation. While vertical transmission of fungal endophytes is well documented (e.g. *Epichloë* and *Neotyphodium* in grasses) (Saikkonen et al. 2002; Tintjer et al. 2008; Wiewiora et al. 2015; Gagic et al. 2018), vertical transmission of bacterial endophytes have also been suggested with growing evidence. The same genera were detected in successive generations of rice and switchgrass (Mukhopadhyay et al. 1996; Gagne-Bourgue et al. 2013). In *Crotalaria pumila*, a pioneer plant in metal-contaminated soils, the seed assemblages are similar across generations (Sanchez-Lopez et al. 2018). In maize, long-term conservation was found in seed endophytic community. A core microbiota was identified in maize seeds from wild ancestors and modern cultivars, suggesting that some groups of bacterial endophytes are conserved across generations despite human selection and cross-continental migration (Johnston-Monje and Raizada 2011). Seeds of genetically related maize hybrids have been found to host similar bacterial taxa. A similar profile of endophytic community was also observed in parental and offspring maize seeds (Liu et al. 2012). It has been suggested that endophytes that are beneficial to the host under a particular circumstance (e.g., abiotic stress) may be passed down to the offspring through seeds (Truyens et al. 2013). However, more direct evidence of vertical transmission is still lacking. Strain-level information is in need besides the 16S rRNA sequence analysis.

1.3 Establishment of root endophytic community

Roots are the primary site for land plants to interact with microbes, as soil is the largest reservoir of microbial diversity (Sanchez-Canizares et al. 2017). Roots provide a very attractive, nutrient-rich niche for microbes by releasing organic compounds. In return, root associated microbes could promote plant growth and outcompete invading pathogens (Berg et al. 2016). The root microbiome is often analogous to human gut microbiome, as they are similar in health and nutrient uptake functions (Hacquard et al. 2015).
Rhizosphere, the narrow region of soil directly influenced by root secretions and associated microorganisms, is known as a hotspot of microbial activity (Berendsen et al. 2012). Although it is not completely understood how they overcome plant defense reactions, a subset of the rhizosphere bacteria could attach to the rhizoplane and gain entry into the root interior (Bulgarelli et al. 2013) (Figure 1-4). Bacteria can enter plants at root hairs, elongation zones, root tips and at emergence sites of secondary roots (Compant et al. 2010; Reinhold-Hurek and Hurek 2011). Some endophytic bacteria can secret cell wall degrading enzymes to facilitate their entry and spread within the plant tissues (Turner et al. 2013). In roots of the

leguminous plant *Robinia pseudoacacia* L., the endophyte *Bacillus subtilis* caused morphological changes in the root hair and colonized the plant through infected root hairs (Huang et al. 2011). The endophytic bacterium *Burkholderia phytofirmas* PsJN initially colonized the root surface of grapevine plantlets and was eventually found in the root hair zone, the root tips and the branching zones of the secondary roots (Compant et al. 2008). The colonization pattern of bacteria might also depend on the host plant. While root hairs are generally favored by *Bacillus amyloliquefaciens* FZB42 in both *Zea mays* and *A. thaliana*, this bacterium colonizes tips of primary roots in *A. thaliana* but not in *Z. mays* (Fan et al. 2012).

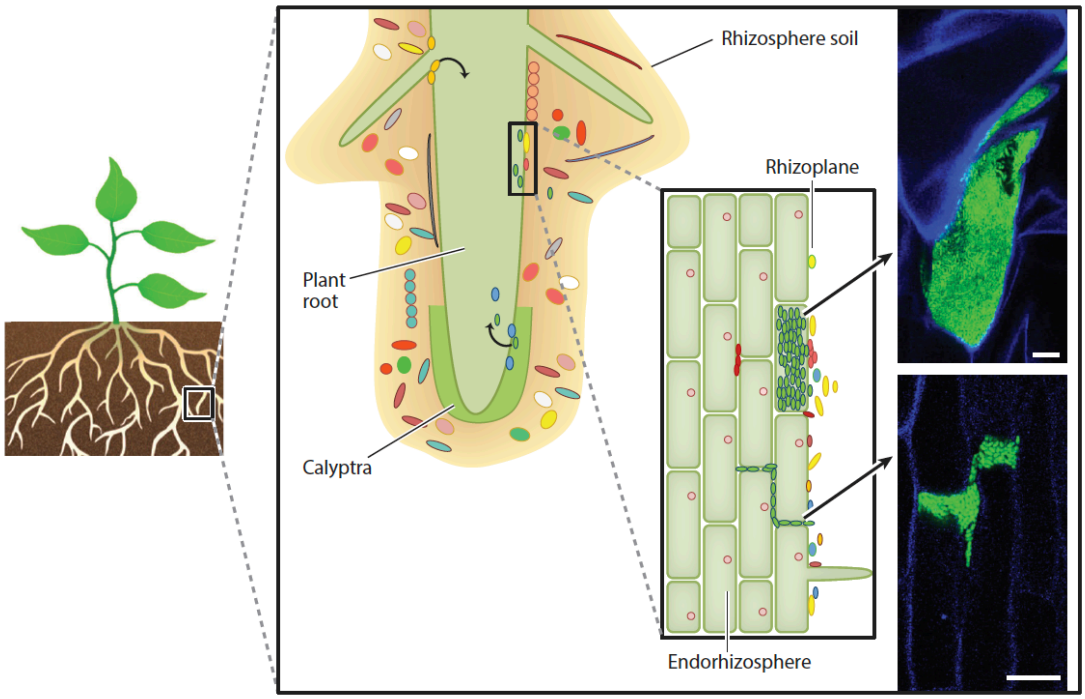


Figure 1-4 Roots with surrounding rhizosphere soil, rhizoplane and endorhizosphere compartments. Confocal laser scanning micrographs show dense intracellular (top) and intercellular (bottom) colonization of roots by endophytic bacteria. Scale bars represent 15 µm (Reinhold-Hurek et al. 2015).

The microbial diversity and richness decline sequentially from bulk soil to the rhizosphere, rhizoplane, and endosphere (Bulgarelli et al. 2013). Each compartment represents a unique ecological niche and hosts different microbial communities. Two comprehensive studies of *A. thaliana* root microbiome found that Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria were enriched in the root compartment compared with bulk soil, whereas Acidobacteria, Verrucomicrobia, and Gemmatimonadetes were depleted (Bulgarelli et al. 2012; Lundberg et al. 2012).

The composition of root endophytes is largely determined by the soil type. Pronounced effects of soil on the root microbiota have been reported for the model plant *A.thaliana* as

well as for various crops (Bulgarelli et al. 2012; Lundberg et al. 2012; Edwards et al. 2015; Yeoh et al. 2017).

The plant genotype effect on the microbial communities has been reported repeatedly. Root bacterial community is correlated with host phylogeny (Schlaeppi et al. 2014). Greater variation in root microbiota has been found between distantly related plants, while genetically related plants seem to have a more similar endophytic profile (Bouffaud et al. 2014; Yeoh et al. 2017; Fitzpatrick et al. 2018). Plant developmental stages also influence the root community assembly. In a recent study, the root microbiota was found to be highly dynamic during the vegetative phase and then stabilized compositionally in the remaining lifetime of rice (Edwards et al. 2018). Compositional changes have been observed in the root microbiome between domesticated plants and their wild relatives, such as barley (Bulgarelli et al. 2015), maize (Bouffaud et al. 2014), Agave (Coleman-Derr et al. 2016) and lettuce (Cardinale et al. 2015).

Plants use root exudates as chemical cues to monitor and interact with their surroundings (Badri and Vivanco 2009). The plant host specificity in terms of plant microbiome is very likely due to the differences in root exudation patterns. Root exudate composition also changes during development and in response to environmental condition. Gas chromatography-mass spectrometry (GC-MS) on root exudates of *A. thaliana* has shown that the levels of sugars and sugar alcohols decreased through plant development, whereas the levels of amino acids and phenolics increased over time (Chaparro et al. 2013). Compounds in root exudates can attract and select preferred microorganisms, and are thus an important driving force in the assembly of root microbial communities (Sasse et al. 2018). For instance, changes in root exudation of *A. thaliana* due to mutation of an ABC transporter gene has been reported. The root exudates, with increased phenolics and fewer sugars than the wild type, led to a relatively higher abundance of potentially beneficial bacteria (Badri et al. 2009). Root exudation is also linked with plant defense response. Exogenous treatment with Jasmonic acid has recently been shown to alter root exudates profile and the composition of root-associated bacterial communities (Carvalhais et al. 2013). Likewise, mutants disrupted in different branches of the jasmonate pathway resulted in distinct exudation patterns compared with the wild type (Carvalhais et al. 2015).

The plant immune system is of particular importance in the establishment of root endophytic community. Currently, two key interconnected branches of the immune system are recognized. One branch recognizes and responds to molecules common to many classes of

microbes, referred to as pathogen-associated (or microbe-associated) molecular patterns (PAMPs/MAMPs). The second branch responds to pathogen virulence factors, which may be secreted as effector proteins into the apoplast or directly into the cytoplasm of host cells to suppress the defense response (Jones and Dangl 2006).

A recent study showed that the plant defense-related hormone Salicylic acid (SA) fine-tuned the assembly of the microbiome in the root endosphere of *A. thaliana*. By analyzing the endophytic root microbiomes of SA biosynthesis and signaling mutants, it was found that SA modulates colonization of the roots by specific bacterial families (Lebeis et al. 2015). In wheat, the presence of Jasmonic acid (JA) changed the structure of microbial communities of the root endosphere. Most of the enriched taxa caused by JA signaling were related to phytopathogen-suppressing, nutrient mobilization, and plant growth promotion. Moreover, the effect was only observed in the endosphere but not in the rhizosphere (Liu et al. 2017). Intriguingly, nutrient deficiency and other abiotic stress responses are shown to cross communicate with plant immunity. A set of *A. thaliana* mutant plants in phosphate accumulation pathways showed a distinctive assembly of communities. It was demonstrated that the master transcriptional regulator of the phosphate starvation response, PHR1, negatively regulates immunity by binding to the promoters of SA- and JA- response genes. Thus, nutritional stress was prioritized over plant defense (Castrillo et al. 2017).

1.4 Genomic insights into bacterial endophytes

Bacterial endophytes have evolved genes that enable them to colonize host plant and adapt to plant environments. Bacterial isolates sequencing and metagenomics of endophytic community have shed light on the genomic features of endophytes. The major traits frequently reported in endophytes include chemotaxis, twitching motility, plant cell wall degradation, detoxification of reactive oxygen species, iron acquisition and storage, quorum sensing, protein secretion and phytohormone production (Sessitsch et al. 2012). Beneficial functional capacities such as nitrogen fixation, 2,3-butanediol, acetoin, and indole acetic acid (IAA) production, have also been reported in bacterial endophyte genomes (Reinhold-Hurek and Hurek, 2011). (Figure 1-5).

A few characteristics have been investigated by mutational analyses with well-described bacterial endophytes. Microbial surface structures, especially the rhamnose biosynthesis and incorporation into the lipopolysaccharide (LPS) had an impact on *Herbaspirillum seropedicae* to attach and endophytically colonize maize root (Balsanelli et al. 2010; Mitra et al. 2016). Type IV pili were found essential for the grass endophyte *Azoarcus sp*. BH72 to

form biofilm on the surface of rice roots (Timmusk and Wagner 1999). A mutant of *Azoarcus sp.* BH72, which still produced pili but lost twitching motility, was particularly impaired in the endophytic establishment, though it could still form microcolonies on the rice root surface (Bohm et al. 2007). Similarly, a mutant lacking flagella was significantly affected in the endophytic colonization (Buschart et al. 2012). Although flagellins are common pathogen/microbe associated molecular patterns (PAMPs/MAMPs) eliciting defense responses, flagellar apparatus are common in endophytes and are suggested to mediate endophytic competence (Reinhold-Hurek et al. 2015). The flagellin of the beneficial endophyte *Burkholderia phytofirmans* appears to escape the grapevine immune system mediated by the FLS2 receptor (Trda et al. 2014). Plant-polymer degrading enzymes such as cellulases and pectinases have also been suspected to play a role for internal colonization. Systemic spreading into the rice shoot could no longer be detected for an endoglucanase mutant of *Azoarcus* sp. BH72. Ingress into root epidermis cells was reduced as well (Reinhold-Hurek et al. 2006). In *Burkholderia phytofirmans*, mutations that decreased the production of the quorum-sensing autoinducers *N*-acyl-homoserine lactones reduced both epi- and endophytic root colonization (Zuniga et al. 2013). Among the metabolic adaptations, bacterial ethanol dehydrogenases play a role in the colonization of rice roots by *Burkholderia phytofirmans* PsJN (Tseng et al. 2009). The reactive oxygen species scavenging enzymes are also essential in the endophytic lifestyle. The mutants of *Gluconacetobacter diazotrophicus* PAL5 incapable of producing superoxide dismutase and glutathione reductase were unable to efficiently colonize the roots (Alqueres et al. 2013).

Protein secretion plays a central role in modulating interactions of endophytes and host plants. Type III, type IV, and type VI secretion systems are of particular interest, as they involve a translocation unit that allows direct injection of proteins into the cytoplasm of host cells (Tseng et al. 2009). Type III secretion systems (T3SS), which are commonly recruited in pathogenic and symbiotic interactions, are extremely rare or incomplete among endophytes (Sessitsch et al. 2012). For example, the endophyte strain *Burkholderia phytofirmans* PsJN has all the components of T3SS, except the needle-forming protein (Reinhold-Hurek and Hurek 2011). It has been proposed that endophytes might be regarded as disarmed pathogens (Reinhold-Hurek and Hurek 2011). In contrast, type VI secretion system (T6SS) is commonly found in endophytes and was also abundantly represented in the metagenome of rice root endophytes, indicating that it might play an important role in the host-microbe interaction (Reinhold-Hurek et al. 2015).

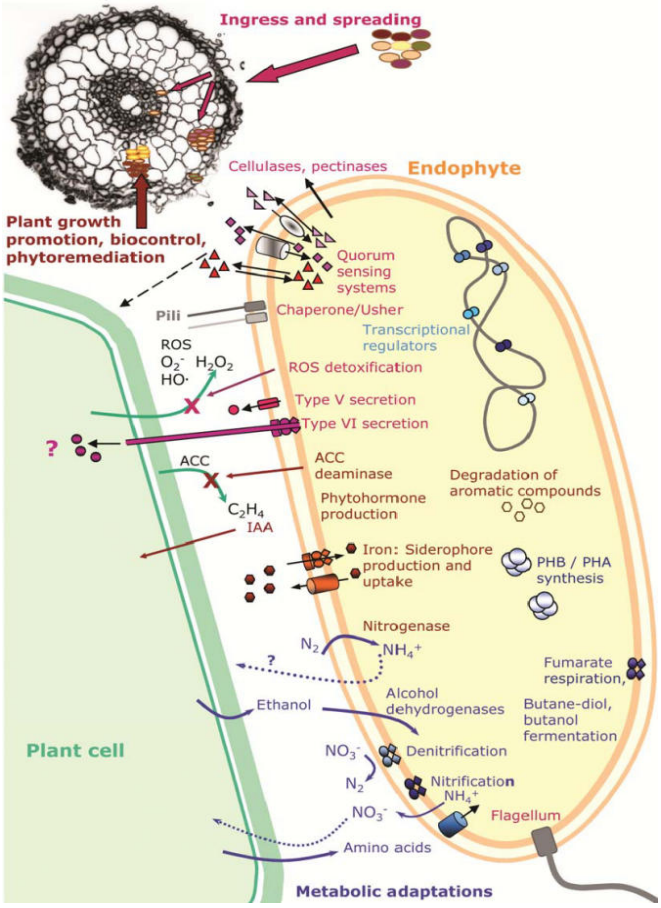


Figure 1-5 Reconstruction of rice-endophyte interactions inferred from gene content analyses of the rice endophyte metagenome (Sessitsch et al. 2012).

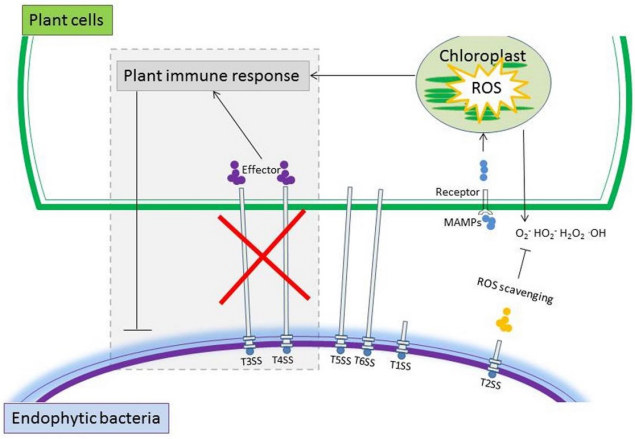


Figure 1-6 Schematic representation summarizing typical secretion systems that may be employed by endophytic bacteria to cope with the plant's immune system. (Liu et al. 2017)

Many researchers have tried to figure out the unique features that determine the establishment of bacteria endophytic lifestyles. Comparing bacterial endophytes with their non-endophytic counterparts, it was found that genomes of plant-associated bacteria encode more carbohydrate metabolism functions and fewer mobile elements than related non-plant-associated genomes do (Levy et al. 2018). Genome comparison of endophytes, plant symbionts and pathogens indicates that discriminative properties are the responsiveness to

environmental cues, nitrogen fixation, and protection against reactive oxygen and nitrogen species (Hardoim et al. 2015). The study comparing *Burkholderia phytofirmans* PsJN and several other strains did not find unique gene cluster that could be exclusively linked to endophytes. Instead, they demonstrate a wide spectrum of endophytic lifestyles in terms of many aspects, such as iron acquisition and quorum sensing (Mitter et al. 2013).

In a recent study, plant-associated protein domains were identified as a common genomic feature of endophytes. Proteins containing these domains were predicted to be effector proteins of bacteria, whereas plant proteins carrying these domains belonged to the intracellular innate immune receptors. Thus, these proteins may mimic plant proteins and interfere with plant immune system (Levy et al. 2018). In previous literature, a set of eukaryote-like protein domains have been reported in the genome of *Pantoea ananatis* recovered from maize seeds (Sheibani-Tezerji et al. 2015) and *Methylobacterium extorquens* DSM13060, an intracellular scots pine shoot symbiont (Koskimaki et al. 2015).

1.5 Harnessing beneficial endophytes: toward sustainable agriculture

Plant microbiome is one of the key determinants of plant health and productivity (Turner et al. 2013). Beneficial endophytes could be exploited as biofertilizer, growth stimulants, and biocontrol agents replacing chemical fertilizers and pesticides, thus supporting sustainable agriculture. Furthermore, endophytes that possess pollutant-degrading and/or plant growth-promoting activities can assist phytoremediation to remediate soil and water polluted by toxic organics and/or metals (Ryan et al. 2008).

The best-studied plant growth-promoting genera include *Azospirillum*, *Azoarcu*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pantoea*, and *Pseudomonas* (Miliute et al. 2015). The proposed mechanism by which endophytes stimulate plant growth includes enhanced nutrient acquisition, plant hormone production and ACC (1-aminocyclopropane-1-carboxylate) deaminase (Rosenblueth and Martinez-Romero 2006). Recently, the emission of volatiles was discovered as a novel mechanism of endophytes to promote plant growth. Researchers demonstrated that 2,3-butanediol and acetoin significantly enhance the growth of *A. thaliana* (Ryu et al. 2003). Some endophytes can produce vitamins, especially B-group vitamins (Marek-Kozaczuk and Skorupska 2001). Vitamin B_{12} produced by endophytic bacteria is suggested to benefit plants, as revealed in studies of algae and bryophytes (Croft et al. 2005; Grant et al. 2014). Plants inoculated with endophytes had increased chlorophyll content and photosynthesis activity (Li et al. 2014). Microbial photosynthetic activity or at

least the presence of relevant genes has been shown for various plant-associated Proteobacteria such as *Bradyrhizobium* spp. (Giraud et al. 2007).

Endophytes can also alleviate the abiotic stress of plants. Inoculation with bacterial endophyte *Burkholderia phytofirmans* strain PsJN alleviated the drought stress in maize and wheat (Naveed et al. 2014; Naveed et al. 2014). The root fungal endophyte *Pirifomospora indica* confers drought tolerance in *A. thaliana* by stimulating the expression of drought stress-related genes (Sherameti et al. 2008). Tomato plants inoculated with two psychrotolerant *Pseudomonas* strains were able to cope better with chilling stress. Less membrane damage, improved antioxidant activity in leaf tissues, and higher expression of cold acclimation genes were observed in endophyte-inoculated plants (Subramanian et al. 2015).

Endophytic microorganisms with biocontrol properties can protect plants from pathogens and herbivores. Endophytes may act directly by niche occupation, producing antimicrobial compounds against pathogens and herbivores or by producing signal components interfering with pathogens (Card et al. 2016). Bacterial endophytes can also elicit induced systemic resistance (ISR) of plants, leading to the enhanced defense of plants against pathogens (Pieterse et al. 2014). For instance, *Pseudomonas fluorescens* PICF7, a native olive root endophyte, is able to trigger a broad range of defense responses. It is an effective biocontrol agent against *Verticillium* wilt of olive (Cabanas et al. 2014). Endophyte-elicited ISR was also observed in cucumber inoculated with *Bacillus pumilus* INR7 with reduced severity of angular leaf spot, cucurbit wilt and the infestation of cucumber beetles (Yi et al. 2013).

Many endophytes have shown the capacity of xenobiotic degradation. This natural ability to degrade these xenobiotics is investigated concerning improving phytoremediation. Researchers showed that plants grown in soil contaminated with xenobiotics naturally recruited endophytes with the necessary contaminant-degrading genes (Siciliano et al. 2001). In the field sites contaminated with petroleum compounds, genes encoding for petroleum compound degrading were more prevalent in endophytic strains than in the rhizosphere communities (Siciliano et al. 2001). In another study, a bacterial endophyte *Pseudomonas putida* VM1450, capable of degrading herbicide, reduced the accumulation of the herbicide into plant tissues, when inoculated to pea plants (Germaine et al. 2006). Upon exposure to heavy metals, endophytes could enhance the tolerance of plants to high metal concentrations (Ma et al. 2016).

Recently, the SynBiotic (combining prebiotic and probiotic treatment) approach for crop cultivation was proposed by joining breeding and introducing beneficial microbes on or in seeds (Figure 1-7) (Berg and Raaijmakers 2018). This should facilitate the breeding of new cultivars supporting beneficial microorganisms. Potential promising routes have been reported to engineer plant microbiome for optimizing the plant phenotype of interest (Qin et al. 2016). It was shown that plant microbiome and traits could be modified by introducing beneficial endophytes into progeny seeds (Mitter et al. 2017). In another study, microbiome engineering was demonstrated in seeds of the Styrian oil pumpkin, a crop with a short breeding history (Adam et al. 2018).

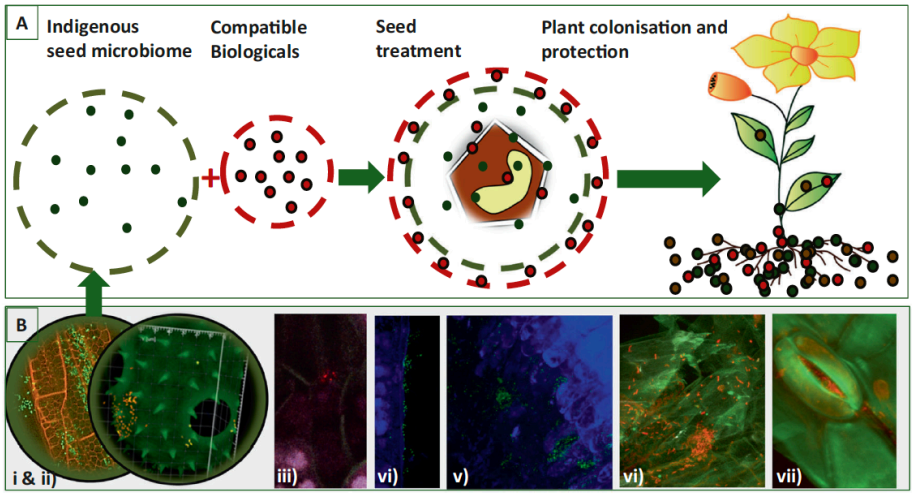


Figure 1-7 (a) showing the concept of compatible biologicals for crops. (b) visualizing endophytes by in situ hybridization and confocal laser scanning microscopy (i) in the endosphere, (ii) on pollen, (iii) naturally occurring in seeds, (iv) after seed treatment within seed on the cotyledon, (v) after seed treatment within seed on the root hypocotyl embryo, (vi) in the rhizosphere and (vii) phyllosphere after seed treatment. Seeds were treated with *Serratia plymuthica* (Berg and Raaijmakers 2018)

A better understanding and application of endophytes is of great importance for plant breeding and plant biotechnology (Finkel et al. 2017). The successful re-integration of microbial functions may contribute greatly to more sustainable agriculture.

1.6 Aims and hypotheses

Seed associated microorganisms have significant impacts on seed quality and plant fitness. However, current knowledge on the ecology of seed-borne endophytes is rather limited. The composition and function of seed-borne endophyte have yet to be characterized. The influence of the seed-borne endophytes on plant microbiota assembly remains elusive. In this thesis, we used barley (*Hordeum vulgare*) as a model plant to study the endophytes inhabiting seeds and roots. The main hypotheses are

I. plant genotype/cultivar shape the composition of bacterial seed endophytes, as with the rhizosphere and phyllosphere
II. the functions of seed-borne endophytes in different barley cultivars are similar although their taxonomic composition may differ
III. seed-borne endophytes greatly influence the composition of root microbiome but will be substituted by bacterial populations in the rhizosphere during plant development
IV. plants will perform better under drought stress in presence with natural soil microbiome compared with autoclaved soil with a disturbed microbiome
V. due to the disturbance of soil microbiota by autoclavation, an enrichment of seed-borne endophytes will be observed in roots of barley plants.

To test these hypotheses, three experiments have been conducted.

I. Five barley cultivars were grown in the soil-free systems (axenic) and soil-based systems. The seed and root associated bacterial community were analyzed with amplicon sequencing. This experiment contributes to working hypothesis I and III.
II. Two barley cultivars were grown in sterile sand mixture under controlled conditions in the greenhouse. Bacterial cells from surface-sterilized roots were analyzed with metagenome sequencing. This experiment contributes to working hypothesis II.
III. Barley plants were grown in natural and autoclaved soil respectively. The drought was imposed on plants two months after regular watering. Plant performance of barley grown in different soils was measured. Bacterial and fungal root endophytes of barley in different soils and treatments were analyzed using amplicon sequencing. This experiment contributes to working hypothesis IV and V.

Results of Experiment I are published.
Luhua Yang, Jasmin Danzberger, Anne Schöler, Peter Schröder, Michael Schloter and Viviane Radl (2017) Dominant Groups of Potentially Active Bacteria Shared by Barley Seeds become Less Abundant in Root Associated Microbiome. *Frontiers in Plant Science*. 8:1005.

In this publication, I was involved in the experimental design. I conducted the experiment of growing barley in soil-based systems, sampling and sequencing. I performed bioinformatic and statistical analysis and wrote the draft of the manuscript.

Manuscripts of the other two experiments are in preparation.

2. Materials and methods

2.1 Experimental design

In order to verify the proposed hypotheses and answer the research questions, three different experiments were conducted. We used barley as the model plant to study seed and root endophytes. Seeds used in this project were obtained from two companies. Alexis and Barke were provided by Saatzucht Breun GmbH & Co. KG (Herzogenaurach, Germany). Marthe, Salome and Simba were supplied by Nordsaat Saatzucht GmbH (Langenstein, Germany). The details of each experiment were described as followings.

2.1.1 Experiment I: the influence of barley seed microbiome on the composition of root endophytes

In this experiment, the major aims are: (a) to characterize barley seed and root endophytes and identify the influencing factors, (b) to investigate the role of seed endophytes as 'first inoculum' in root microbiome and the stability of this 'first inoculum' during plant development.

Five modern cultivars of barley were selected, namely Alexis, Barke, Marthe, Salome, and Simba. We used two systems to grow barley, which are i.) axenic soil-free systems without external microbes, and ii.) soil-based systems, where microbes from the rhizosphere can also colonize the root interior. Plants were sampled at the seedling stage and booting stage according to Zadoks decimal code for the growth of cereals (Zadoks et al., 1974).

DNA and RNA were co-extracted from surface-sterilized seeds and roots. Bacterial communities associated with seeds and roots were characterized using amplicon sequencing. We used DNA based sequencing to study the resident groups and rRNA based sequencing to study the active community, as the presence of rRNA is indicative of protein synthesis and has been widely applied to characterize active microbes (Blazewicz et al. 2013). In this experiment, the rRNA based sequencing was applied to all the samples, while DNA based sequencing was applied to a subset of samples, including seeds and roots grown in axenic systems. Both cultivar and growth stage effects were examined. Bacteria associated with seeds and roots were compared to study the role of seed endophytes in the composition of root microbiome. The experimental design and procedures are represented in the flowchart in Figure 2-1.

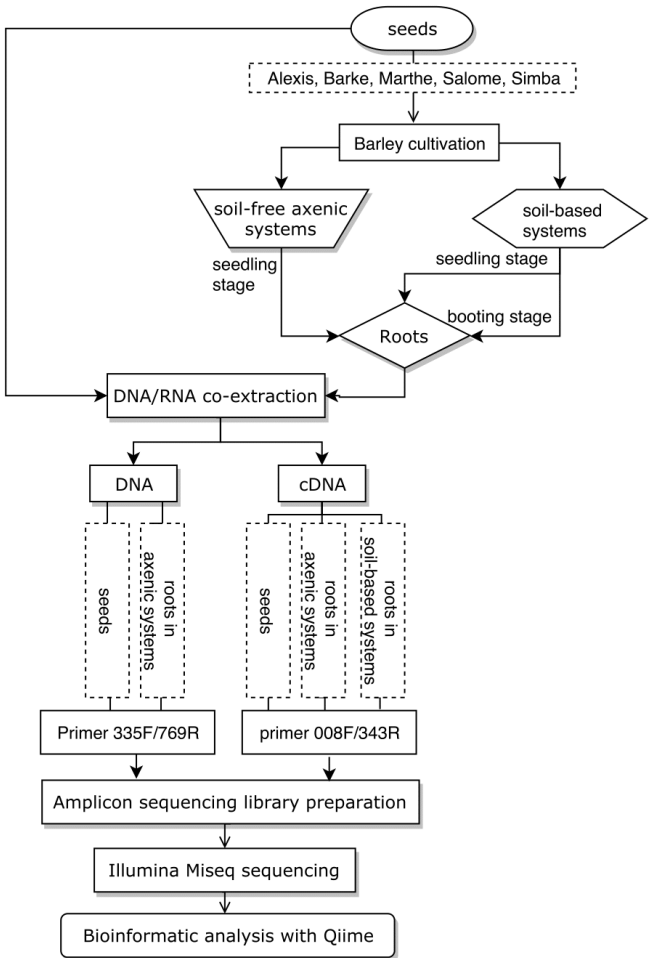


Figure 2-1 The flowchart of experimental design and procedures in Experiment I

2.1.2 Experiment II: functional potentials of seed-borne endophytes in barley root microbiome

In this experiment, our aim is to investigate the functional potentials of root endophytes which are seed-borne. We hypothesize that different barley cultivars have similar functions although they may differ taxonomically.

To get root endophytes originated from seeds, we grew barley cultivars Barke and Salome in sterile sand and vermiculite mixture under controlled conditions in the greenhouse. The metagenome sequencing of root endophytes is challenging, because the extracted DNA is largely plant-derived. Host DNA can quickly drown out microbial reads. The small fragments of metagenomic reads make it difficult to differentiate microbial and host-derived genes in bioinformatic analysis.

To get rid of plant DNA interference, we pooled surface-sterilized roots for endophytic bacteria concentration. Bacterial cells were concentrated with a series of differential centrifugations followed by a Nycodenz density gradient centrifugation using a modified protocol (Ikeda et al. 2009). The efficiency of the bacterial cell enrichment was assessed in

our lab with DAPI staining, qPCR and sequencing (Bigott 2017). With the DNA extracted from the bacterial pellets, further metagenome sequencing was carried out on Illumina Miseq platform to study the functions of seed-borne endophytes in roots. The experiment was carried out three times. As a result, each cultivar has three libraries sequenced.

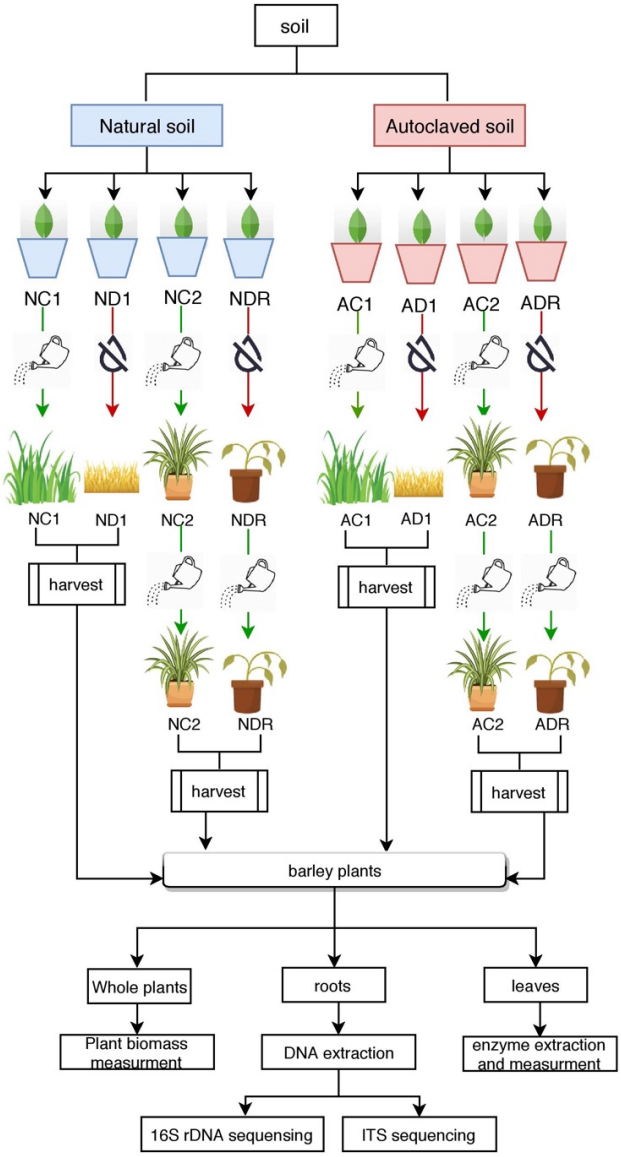


Figure 2-2 Flow chart of the experimental design in chapter 3

(The green line indicates watering, while the red line indicates water withholding.)

2.1.3 Experiment III: the role of soil microbiome and seed-borne endophytes in barley drought response

The main objectives are to study if microbes could help plants cope with drought and to investigate the drought stress response of root endophytes. The experimental design is illustrated in Figure 2-2. Barley cultivar Barke was used as our model plant. In this experiment, we grew barley in natural soil and autoclaved soil respectively.

In the first two months, all barley plants were treated the same. Plants were irrigated with tap water twice per week until two months. Two months after seeds planting, we imposed drought to plants grown in the natural soil designated as ND1 and NDR, as well as plants grown in the autoclaved soil labelled as AD1 and ADR. Corresponding control samples, NC1, NC2, AC1 and AC2, were watered regularly as before. Plants NC1, ND1, AC1, and AD1were harvested one week after drought, as the plants under drought (ND1 and AD1) showed severe symptoms of dehydration. Plants NDR and ADR were re-watered after the one-week's drought, while the corresponding controls NC2 and AC2 were continuously under regular irrigation. Two weeks later after re-watering, all plants NC2, NDR, AC2 and ADR were harvested.

Water content, plant biomass and peroxidase activity of barley grown in different soils were measured as indicators of plant performance under drought stress. DNA was extracted from surface sterilized roots. Endophytic bacterial and fungal communities in different soils and treatments were analyzed using amplicon sequencing.

2.2 Seeds surface sterilization and germination

Before germination, we surface sterilized the seeds with chemical treatment using ethanol and NaClO. This method was selected because a rigorous microscopic comparison showed that it is more efficient in removing surface microbes than commonly used ultra-sonication and shaking (Reinhold-Hurek et al. 2015). Seeds were immersed in 1% Tween 20 for 2 minutes and 70% ethanol for 5 minutes. After washing for 5 times with sterile water, seeds were treated with 2% NaClO for 20 minutes, followed by thorough washing with sterile water for 5 to 6 times.

The success of the surface sterility for seeds was checked by fluorescence in situ hybridization (FISH) using probes Eub-335-I, Eub-335-II and Eub-338-III (Metabion, Germany) as described in the literature (Spohn et al. 2015) and plating on R2A agar plates for 24 h at 23 °C in dark. Surface sterilized seeds were germinated on wet paper in Petri dishes in the dark for 3 days at 30 °C.

2.3 Barley cultivation and harvest

2.3.1 Growing barley in the climate chamber

In experiment I, we grew five cultivars of barley (Alexis, Barke, Marthe, Salome, and Simba) in axenic systems in the climate chamber (Figure 2-3-a). The axenic systems were prepared as the followings: beakers (250 ml) were filled with 185 g glass beads (⌀1.7-2.1 mm, ROTH,

Germany) and 45 ml MS media (Duchefa Biochemie bv, The Netherlands), and 6 germinated seeds were put in the glass beads and covered with another beaker. All beakers, glass beads and media were sterile. We then sealed the complete system with Parafilm. Five replicates (each consisting of 6 seeds) were used per cultivar. Plants were grown in the climate chamber under controlled conditions (23 °C/14 h, 15 °C/10 h, and 65% humidity).
We used the Zadoks decimal code (Zadoks et al., 1974) for the growth stage scale and determined our sampling time accordingly. Barley plants grown in axenic systems were sampled 8 days after sowing the seeds when they were in seedling stage (Zadoks code Z13). Attached beads were removed by shaking and washing thoroughly. Surface sterilization and the sterility checking of roots were performed as described above for the seeds. Roots were shock frozen with liquid nitrogen and stored at -80 °C.

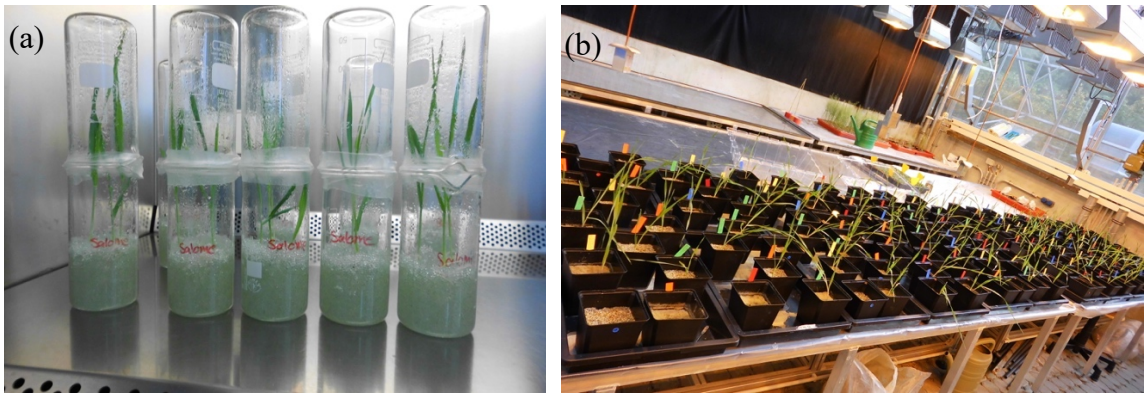


Figure 2-3 Pictures showing barley cultivation in (a) axenic systems in the climate chamber and (b) soil-based systems in the greenhouse

2.3.2 Growing barley in the greenhouse

Experiment I

We grew five cultivars of barley (Alexis, Barke, Marthe, Salome, and Simba) with the agricultural soil collected from the top layer of field A15 in Scheyern Research Farm (Scheyern, Germany), which has a sandy texture. The soil was sieved through a 2 mm mesh to remove large stones, plant debris, and earthworms. Sieved soil was filled into pots which were 13 cm high, with the top square of 13 x13 cm and bottom square of 9.6 x9.6 cm. Germinated seeds were sown in pots filled with soil. Each pot contained one well-germinated seed. Five replicates were prepared for each cultivar. Pots with different cultivars were placed randomly. The plants were grown in the greenhouse under controlled conditions with 12 h light at 20 °C and 12 h dark at 16°C and were watered with tap water twice a week (Figure 2-3-b).
Barley plants were sampled at two time points respectively based on Zadoks decimal code (Zadoks et al., 1974): 2 weeks after planting (seedling growth, Z13) and 10 weeks after

planting (booting, Z41). Plants were transferred to the lab on ice immediately after soil was roughly shaken off. Roots were thoroughly washed, surface sterilized, shock frozen and stored as described above.

Experiment II

Well-germinated seeds were transferred to pots in the greenhouse. The clean pots were wiped with 70% ethanol carefully and filled with a sterile mixture of sand and vermiculite (volume ratio=1:1). The mixture was autoclaved at 134 °C for 120 min twice with an interval of one week. Each pot contained five well-germinated seeds. In total, 30 to 35 pots were prepared for each cultivar. The plants were grown in the greenhouse under controlled conditions with 12 h light at 20 °C and 12 h dark at 16 °C. Plants were irrigated with autoclaved tap water twice every week. Sterile Hoagland solution (1×) was applied once a week as fertilizer. One unplanted pot with sand mixture served as the negative control.

Plants were harvested after growing for 19-20 days when they were in the seedling stage (Z13). After surface sterilization, fresh roots were used immediately for bacterial cell enrichment described in detail below (section 2.).

The sand mixture in the unplanted pot was also sampled. DNA was extracted from the sand mixture. Real-time quantitative PCR (qPCR) was performed to check the microbial contamination of the sand mixture.

Experiment III

In this experiment, the soil was collected from the area described in Experiment I. Collected soil was sieved through a 2 mm mesh and divided into two portions. One portion of the soil was stored at room temperature, designated as natural soil. The other portion was autoclaved at 134 °C for 2 h twice with an interval of 7 days, designated as autoclaved soil. To confirm the reduction of microbial biomass in the autoclaved soil, cells from both natural and autoclaved soil were extracted using the method described in the literature (Eichorst et al. 2015). The cells were fixed with 4% Paraformaldehyde, stained with DAPI and checked using a fluorescence microscope (Zeiss Axioplan, Germany).

The barley cultivar Barke was used in this experiment. Seeds were surface sterilized and germinated as described in section 2.2. Germinated seeds were transferred to pots filled with natural and autoclaved soil, respectively. Each pot contained two well-germinated seeds. Barley plants in the autoclaved soil were labeled as AC1, AD1, AC2, and ADR, while plants in the natural soil were labeled as NC1, ND1, NC2, and NDR. Each labeling indicates a different treatment. 13 to 15 replicates per treatment were prepared. Pots with different

labeling were placed randomly. The plants were grown in the greenhouse under controlled conditions with 12 h light at 22 °C and 12 h dark at 18°C. In the first two months, all barley plants were treated equally. Plants were irrigated with tap water twice per week. Hoagland solution (1×)(Sigma-Aldrich, USA) was applied as fertilizer once per week.

Two months after planting, we imposed drought to plants grown in the natural soil labeled as ND1 and NDR, as well as plants grown in the autoclaved soil labeled as AD1 and ADR. The drought was applied by ceasing irrigation and leaving the soils dry down progressively. Corresponding control samples, NC1, NC2, AC1, and AC2, were watered regularly as before. Plants NC1, ND1, AC1, and AD1were harvested immediately after the drought period, as the plants under drought showed severe symptoms of dehydration. Plants NDR and ADR were re-watered after the one-week drought, while the corresponding controls NC2 and AC2 were continuously under regular irrigation. Two weeks later after re-watering, plants NC2, NDR, AC2, and ADR were harvested.

Barley plants were taken from the pots carefully to not break the roots system. The attached soil was briefly shaken off. Five randomly selected plants from each treatment were kept intact. They were immediately put on ice and transferred to the lab for biomass measurement. The rest of the plants were cut into leaves and roots with sterile scalpels. The leaves were immediately frozen by liquid nitrogen and stored at -80 °C for further enzyme extraction. The roots were transferred to the lab on ice, washed thoroughly under tap water and surface sterilized as described in section 2.2. After sterilization, the roots were shock frozen in liquid nitrogen and were stored at -80 °C.

2.4 Soil water content, plant biomass, and relative water content measurement

The fresh weight of soil, barley leaves, and roots were measured with balance. The samples were then left in the oven at 105 °C. After drying for 72 h, the dried samples were weighed again. The water content was calculated using the following equation: Mn = ((Ww-Wd)/Wd) x 100, in which: Mn = moisture content (%) of material, Ww = wet weight of the sample, and Wd = weight of the sample after drying.

2.5 Enzyme extraction and measurement

Leaves were grounded in liquid nitrogen using sterile mortar and pestle. Three gram of the fine powder were homogenized with 30 mL extraction buffer (pH 7.8) containing 0.1M Tris, 5mM EDTA, 1% PVP K90, 1% Nonidet P 40 and 5mM DTE at 4 °C for 30 min. After centrifugation at 20,000 × g for 30 min at 4 °C, samples were filtered with Miracloth. The

supernatant was precipitated progressively by adding ammonium sulphate in two subsequent steps. Firstly, ammonium sulphate was added to the supernatant to a concentration of 40% (w/v) and the mixture was centrifuged at 20,000 × g for 30 min at 4 °C. After centrifugation, the supernatant was transferred to a clean beaker and added with ammonium sulphate with a concentration of 80% (w/v). The mixture was then centrifuged at 20,000 × g for 30 min at 4 °C again. Afterwards, the supernatant was discarded carefully. The obtained pellet was re-suspended in 2.5 mL 25 mM Tris/HCl buffer (pH=7.8). Proteins were desalted by chromatography through PD 10 columns (GE Healthcare, UK) and stored at -80 °C for further use. Peroxidase (POX, EC 1.11.1) activity was assayed at 420 nm using guaiacol (4 M per reaction) as the substrate and H_2O_2 (2.56 M per reaction).

2.6 Endophytic bacterial cell enrichment

We enriched the bacterial cells using a protocol modified from a previously described method (Ikeda et al. 2009). Roots from every 10 randomly selected pots were pooled as one sample for enrichment. Three replicates of each cultivar were obtained with the fresh weight of the roots varied from 25 to 38 g.

Sterilized roots were homogenized with 400 ml bacterial cell extraction (BCE) buffer (50 mM Tris–HCl [pH7.5], 1%Triton X-100, 2mM 2-mercaptoethanol) in a blender for one minute with full speed. The homogenization was repeated four times and the blender was cooled on ice for 1 min between each running period. The homogenate was filtered through a double layer of sterilized Mira cloth and centrifuged at 500 g for 5 min at 10 °C.

The supernatant was transferred to new centrifuge tubes and centrifuged for at 5,500 g for 20 min at 10 °C. The supernatant was discarded and the pellets were resolved in BCE buffer to a total volume of 50 ml. The suspension was filtered through a double layer of sterilized Kimwipes and centrifuged at 10,000 g for 10 min at 10 °C. After repeating this step once, the pellet was resuspended in 6 ml 50 mM Tris-HCl [pH 7.5]. The suspension was divided into two aliquots and each was carefully overlaid on 5 ml Nycodenz solution (8 g of Nycodenz dissolved in 50 mM Tris-HCl [pH 7.5]) to a total volume of 10 ml). The density centrifugation was run at 10,000 g for 40 min at 10 °C. The microbial fraction was visible as a white band at the Nycodenz-water interface. The microbial fraction was collected and mixed with the same volume of sterilized distilled water. After centrifugation at 10,000 g for 3 min and removal of the supernatant, the resulted pellet was stored at -20 °C for DNA extraction.

2.7 Nucleic acid extraction

2.7.1 Direct extraction from plant tissues

Surface sterilized seeds and roots were used for nucleic acid extraction. After plating on R2A agar plates for 24 h at 23 °C in dark, the imbibed seeds were grinded using liquid nitrogen and a mortar and pestle. Each sample was composed of six seeds. Root samples were ground into powder using TissueLyzer II (Qiagen, Germany) according to the manufacturer's instruction. 0.1 g of the seed powder and 0.3 g of the root powder were used for a co-extraction of DNA and RNA respectively using Griffiths' protocol (Griffiths et al. 2000). Extraction was performed for each cultivar in five replicates of seeds and three to five replicates of roots. Water served as a negative control and was used for the extraction of nucleic acids in a parallel approach.
The co-extracts were divided into two aliquots. To get cDNA, one aliquot was digested with DNase Max™ Kit (MoBio, USA). Complete DNA digestion was checked and confirmed with real-time quantitative PCR (qPCR). The resulted purified RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA).

2.7.2 Nucleic acid extraction from bacterial cells

DNA from bacterial cell pellets was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Germany). The extraction was performed following the kit protocol with an incubation time for cell lysis of 6-7 hours. The extraction from the kit buffer was performed in a parallel approach and served as the negative control. DNA quantity was assessed with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA).

2.8 Library preparation and sequencing

2.8.1 Amplicon sequencing

In Experiment I, we used ribosomal RNA (rRNA) based amplicon sequencing to study the active groups and DNA for the resident community, as the presence of rRNA is indicative of protein synthesis and has been widely applied to characterize active microbes (Blazewicz et al. 2013). In this experiment, the rRNA based sequencing was applied to all the samples, while DNA based sequencing was applied only to a subset of samples, including seeds and roots growing in axenic systems.
In DNA based sequencing, co-amplification of non-targeted organelle DNA is one of the biggest obstacles in studying bacterial endophytes (Rastogi et al. 2010), because chloroplasts

share high sequence similarities with bacterial 16S rRNA genes (Hanshew et al. 2013). Therefore, we used the primer set 335F/769R targeting the V3 to V4 region of 16S rRNA gene for DNA templates, which was reported to exclude chloroplast amplification (Dorn-In et al. 2015). The cDNA samples, were amplified with the universal primer pair S-D-Bact-0008-a-S-16 (008F) (Muyzer et al. 1993) and S-D-Bact-0343-a-A-15 (343R) (Alm et al. 1996), which covers the V1 and V2 region (Klindworth et al. 2013).

In Experiment III, DNA was extracted from the surface sterilized roots. Amplicon sequencing was conducted to study the bacterial and fungal endophytic community in roots. We used primer pair 335F/769R for the 16S rRNA gene amplification. As to fungi, a mixture of 5 forward primers for ITS2 and a mixture of 4 reverse primers for ITS2 and full ITS (Tedersoo et al. 2015) were used for better coverage.

All the primers were fused with Illumina adaptors. The details of the primers were listed in Table 2-1.

Table 2-1 Primers used for amplicon sequencing

Experi-ments	Target		Primer name	Primer sequence (direction 5′-3′)	References
Exp. I	Bacteria 16S	DNA	335F	CADACTCCTACGGGAGGC	Dorn-In et al., 2015
			769R	ATCCTGTTTGMTMCCCVCRC	Dorn-In et al., 2015
		cDNA	S-D-Bact-0008-a-S-16 (008F)	AGAG TTTGATCMTGGC	(Muyzer et al. 1993) (Klindworth et al. 2013)
			S-D-Bact-0343-a-A-15 (343R)	CTGCTGCCTYCCGTA	(Alm et al. 1996) (Klindworth et al. 2013)
Exp. III	Bacteria 16S	DNA	335F	CADACTCCTACGGGAGGC	(Dorn-In et al. ,2015)
			769R	ATCCTGTTTGMTMCCCVCRC	(Dorn-In et al.,2015)
	Fungi ITS	DNA	ITS3-Mix1 (Fungi)	CATCGATGAAGAACGCAG	Tedersoo et al., 2015
			ITS3-Mix2 (Chytridiomycota)	CAACGATGAAGAACGCAG	
			ITS3-Mix3 (Sebacinales)	CACCGATGAAGAACGCAG	
			ITS3-Mix4 (Glomeromycota)	CATCGATGAAGAACGTAG	
			ITS3-Mix5 (Sordariales)	CATCGATGAAGAACGTGG	
			ITS4-Mix1 (Fungi)	TCCTCCGCTTATTGATATGC	
			ITS4-Mix2 (Chaetothyriales)	TCCTGCGCTTATTGATATGC	
			ITS4-Mix3(Archaeorhizomycetes)	TCCTCGCCTTATTGATATGC	
			ITS4-Mix4 (Tulasnellaceae)	TCCTCCGCTGAWTAATATGC	

Library preparation was accomplished according to the “16S Metagenomic Sequencing Library Preparation” protocol proposed by Illumina Inc., United States. Briefly, polymerase chain reaction (PCR) was performed in triplicates. The 25 µl reaction mixture contained 2.5 µl NEB Next High Fidelity Master Mix (Illumina, USA), 0.5 µl of each primer (10 pmol/µl), 2.5 µl PCR additives/H_2O, 100-200 ng of template DNA/cDNA and ad DEPC water. 3% Bovine serum albumin (BSA) were used as PCR additives for primer set 335F/769R while 36 mM Tetramethyl ammonium chloride (TMAC) were used in fungal reactions instead. No PCR additives were used for the primer pair 008F/343R. The details of the reaction mixture for different primer sets were listed in Table 2-2.

Table 2-2 Components of PCR reaction mixtures for different primer sets

Primer sets	Reaction mixture				
	2.5 µl	2.5 µl	Each 0.5 µl	100-200 ng	Add to 25 µl
335F/769R	3% BSA	NEB Next High Fidelity Master Mix	Forward and Reverse primer (10 pmol/µl)	template DNA/cDNA	DEPC water
008F/343R	H_2O				
ITS mix	36 mM TMAC				

The PCR conditions were as the following: 98 °C for 5 min, followed by 25-30 cycles at 98 °C for 10 s, 60 °C for 30 s and 72°C for 30 s, followed by 72 °C for 5 min. Triplicate amplicons were pooled and purified using Agencourt AMPure XP kit (Beckman Coulter, USA). DNA quantity was assessed with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, USA).

Nextera XT Index Kit v2 (Illumina, USA) was used for amplicon indexing. The indexing PCR was performed using 12.5 µl NEB Next High Fidelity Master Mix, 2.5 µl of each set of indexing primers, 10 ng DNA of the previous PCR products and DEPC water to a total volume of 25 µl. Reactions were kept at 98 °C for 5 min, followed by 8 cycles at 98 °C for 10 s, 55°C for 30 s and 72°C for 30 s, with a final extension step of 10 min at 72°C. All amplicons were purified and quantified as described above. The purified amplicons were then pooled in 4 nM and sequenced on Illumina Miseq platform (Illumina, USA).

2.8.2 Metagenome sequencing

Samples were diluted to equal concentrations in 50 µl. For preparation of the libraries, 50 µl DNA of each sample was mechanically sheared to 400-500 bp fragments using Covaris E220 (Covaris, USA) with the following parameters: incident power 175 W, duty factor 5%, cycles per burst 200, treatment time 35 s, temperature 7 °C, water level 6. The intensifier was selected in the shearing. The fragmented DNA was analyzed with Fragment Analyzer (Advanced Analytical Technologies, USA). The sheared DNA was processed with NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, USA) for end repair and adaptor ligation. All steps were performed according to the instruction manual except for a 10-fold dilution of the adaptors. The size selection of adaptor-ligated DNA was conducted with Agencourt AMPure XP-Kit (Beckman Coulter, USA). The DNA was then amplified with NEBNext Multiplex Oligos for Illumina (New England Biolabs, USA) according to the instruction. Two runs of clean up were performed with Agencourt AMPure XP-Kit (Beckman Coulter, USA) with the ratio of beads to DNA 0.6:1. The purified PCR products were analyzed and quantified using Fragment Analyzer (Advanced Analytical Technologies, USA). The samples were then diluted to 4 nm, pooled and sequenced on Illumina Miseq platform (Illumina, USA).

2.9 Bioinformatic analysis

2.9.1 16S amplicon sequencing analysis

The sequencing analysis was performed with the software QIIME (version 1.9.1) (Caporaso et al. 2010). Adaptors and primers were removed using AdapterRemoval (Lindgreen 2012). Phix contamination was removed using the program Deconseq (Schmieder and Edwards 2011). Reads were merged and filtered by size (amplicon length) and quality (Phred quality score > 2). The sequences were then clustered into operational taxonomic units (OTUs) using open reference strategy based on 97% similarity with GreenGenes Database (13_5 release) as reference. Taxonomy was assigned with RDP classifier (Wang et al. 2007) retrained with GreenGenes 16S rRNA database (13_5 release) (DeSantis et al. 2006). OTUs assigned to chloroplast were filtered out with the command filter_taxa_from_otu_table.py.
The core OTUs of each cultivar were identified as OTUs present in more than 60% samples of each cultivar using the command compute_core_microbiome.py in Qiime. The core OTUs of barley endophytes were defined as shared core OTUs of all the cultivars.

2.9.2 ITS amplicon sequencing analysis

The cleaning and quality filtering steps were the same as described for 16S amplicon sequencing. The ITS region sequences were then extracted using ITSx (version 1.0.11) (Bengtsson-Palme et al. 2013). ITS extraction allowed us to remove the conserved SSU and 5.8S regions, as well as ITS chimeric sequences, resulting in a reliable operational taxonomic unit (OTU) clustering (Sapkota et al. 2015). The resulted ITS2 sequences were then used for OTU clustering with an open reference strategy based on 97% similarity. UNITE database (v7.1) (Koljalg et al. 2013) was used as a reference. Taxonomy was assigned with RDP classifier (v2.2) (Wang et al. 2007). OTUs assigned to Plantae were filtered out with the command filter_taxa_from_otu_table.py.

2.9.3 Metagenome sequencing analysis

Adaptors and primers were removed using AdapterRemoval (Lindgreen 2012). Phix contamination was removed using the program Deconseq (Schmieder and Edwards 2011). The coverage of metagenomes was estimated with Nonpareil, a method that examines the redundancy among the individual reads in the metagenomics data (Rodriguez-R and Konstantinidis 2014). We used Nonpareil because it is independent of assembly and OTU calling, thus avoiding the bias. Processed reads were taxonomically classified using Kaiju (Menzel et al. 2016). The 16s rRNA reads were extracted from the metagenome sequencing

using SortMeRNA (v 2.0) (Kopylova et al., 2012) and were further analyzed with Qiime (v 1.9.1) as described above.
For functional annotation, clean reads were aligned against the KEGG database (June 2011) (Kanehisa and Goto 2000) using DIAMOND (v 0.5.2) (Buchfink et al. 2015) with default settings. The functional assignment was performed using MEGAN (version 5.10.6) (Huson et al. 2016).

2.9.4 Genome reconstruction

The genome reconstruction was conducted with MetaWRAP (v1.05) (Uritskiy et al. 2018). Adaptors and PhiX contamination were removed using BBDUK (v38.19) (http://jgi.doe.gov/data-and-tools/bb-tools/). Sickle version1.33 (Joshi and Fass 2011) was used for quality checking with default setting except for a minimum read length of 100. Reads were assembled using metaSPAdes (Nurk et al. 2017). Metagenomic binning and refinement were performed with MetaWRAP (v1.05) (Uritskiy et al. 2018). The taxonomy of bins was checked by Kraken (Wood and Salzberg 2014). Functional annotation of the reassembled bins was conducted against eggNOG-Mapper (v1.0.3) (Huerta-Cepas et al. 2017).

2.10 Statistical analysis

The statistical analysis was performed in R (version 3.2.1). The alpha diversity was compared with the Kruskal-Wallis test. Unifrac distances were used for the measurement of bacterial beta diversity. Permutational multivariate analysis (PERMANOVA) was conducted with R package “vegan” using the function “Adonis”. Results with p value less than 0.05 were considered statistically significant.

The OTU tables in Experiment III were normalized with R package “DESeq2” (Love et al. 2014). The normalized OTU tables were log2 transformed. The log2fold changes between different treatments were compared using the Wald test. The p-value was adjusted for multiple pairwise comparisons using the Benjamini-Hochberg method. Results with adjusted p values less than 0.05 were considered statistically significant. Permutational multivariate analysis (PERMANOVA) was conducted with R package “vegan”. Unifrac distances and Bray-Curtis dissimilarity were used for the measurement of bacterial and fungal beta diversity respectively.

3. Results

3.1 Barley seed endophytes influence the composition of root microbiome

3.1.1 Barley seed microbiome share a core set of microbial taxa despite cultivar effects

3.1.1.1 Resident groups

In the resident groups of barley seed endophytes detected by DNA based sequencing, we found 7 phyla, 27 families and 43 genera. Taxonomic classification highlighted that barley seed endophytic community was largely dominated by Proteobacteria, Firmicutes, and Actinobacteria, accounting for around 90%, 8% and 1% of total reads respectively (Figure 3-1-1). At family level, Enterobacteriaceae was largely in dominance. Pseudomonadaceae and Paenibacillaceae were also highly abundant.

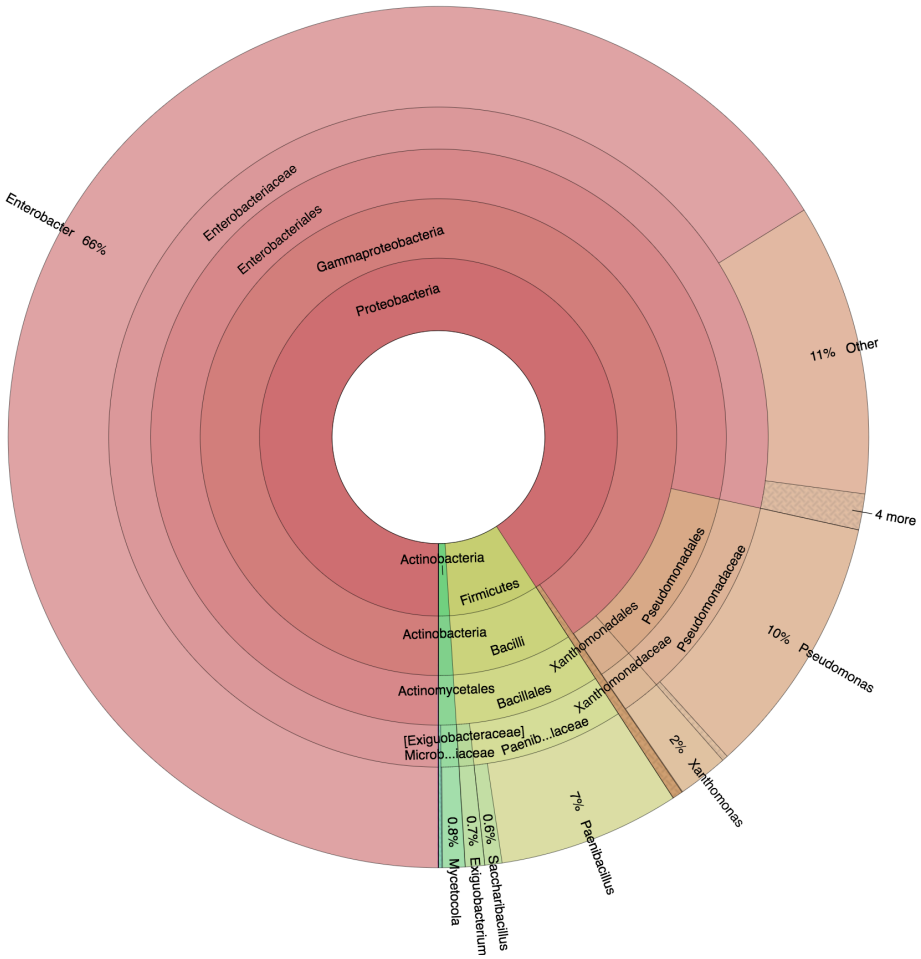


Figure 3-1-1 The taxonomic structure of the resident groups of barley seed endophytes based on 16S rRNA gene (16S rDNA) amplicon sequencing (n=25).
The Krona radial space-filling chart shows the mean relative abundance of bacterial taxa in seeds.

The major genera belonged to *Enterobacter*, *Pseudomonas*, *Paenibacillus*, and *Xanthomonas*. Other genera, like *Erwinia* and *Pantoea*, were also detected but in low abundance (Figure 3-1-2).

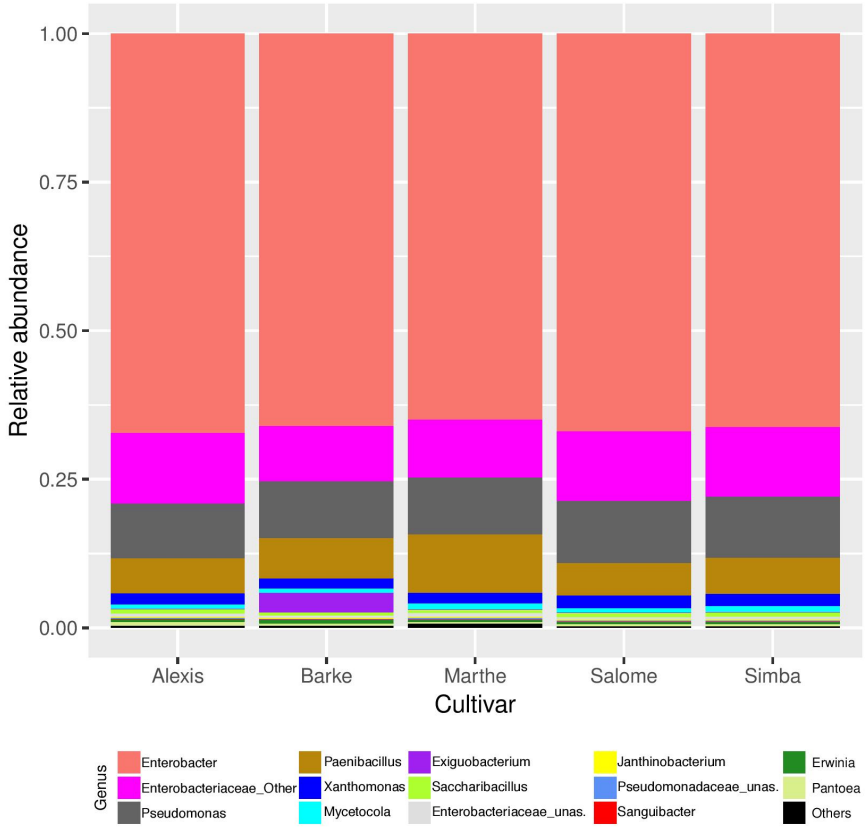


Figure 3-1-2 The major genera of resident bacterial seed endophytes in different barley cultivars based on 16S rRNA gene (16S rDNA) amplicon sequencing (n=5)

Significant differences between cultivars were found in the alpha diversity of resident seed endophytes, where cultivar Marthe showed the lowest diversity (Figure 3-1-3).

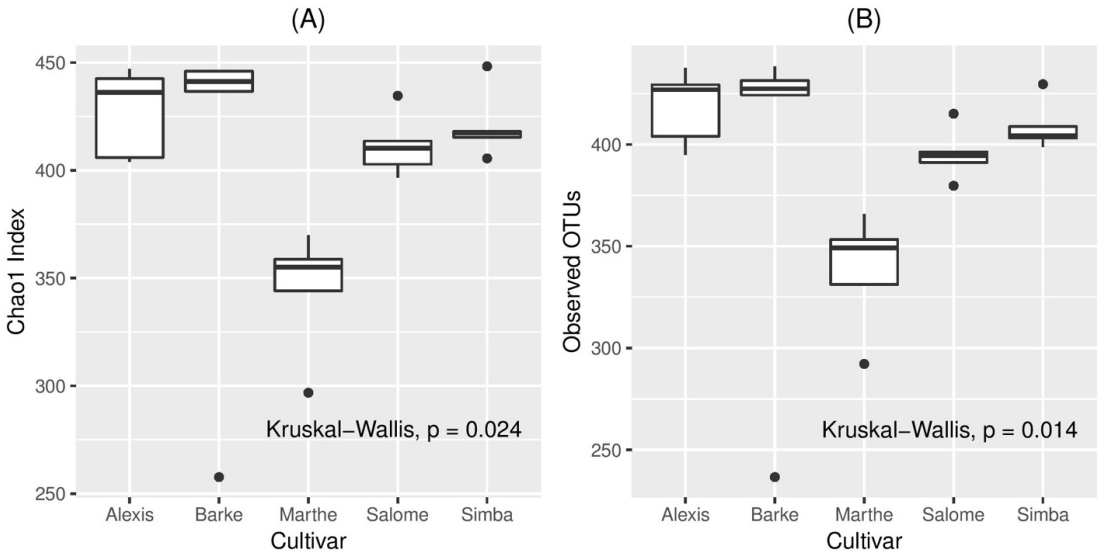


Figure 3-1-3 The (A) Chao 1 index and (B) observed OTUs of resident seed endophytes in five barley cultivars (n=5)

We then carried out principal coordinate analysis (PCoA) based on both weighted and unweighted Unifrac distance metrics (Figure 3-1-4). Differences between bacterial communities across cultivars were indicated by permutational multivariate analysis.

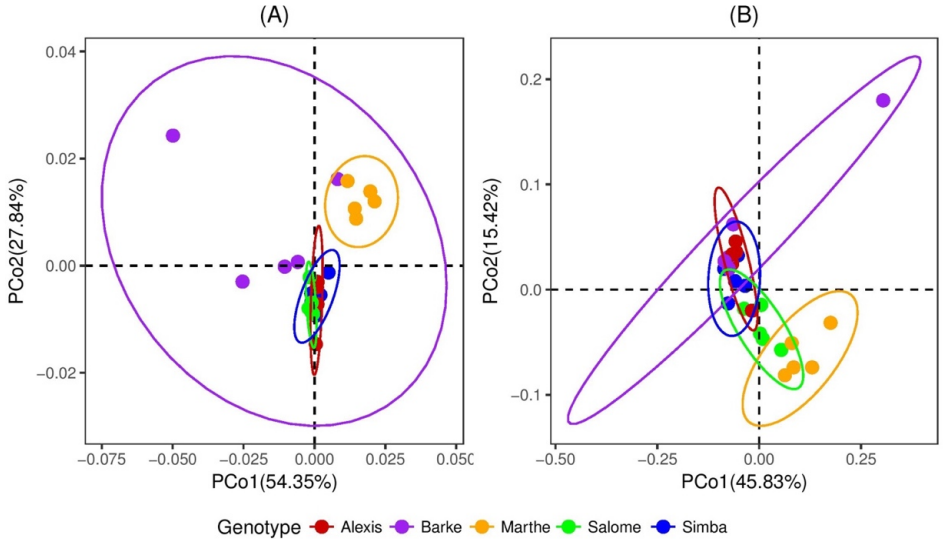


Figure 3-1-4 PCoA plot of resident seed endophytes in five barley cultivars based on (A) weighted (B) unweighted Unifrac distances (n=5)

We further investigated the core OTUs in seed microbiome. The core OTUs of barley endophytes were identified in two steps. First, OTUs present in no less than 60% samples of one cultivar were picked out as core OTUs of the cultivar. The common core OTUs shared by all the cultivars were then considered as the core OTUs of barley endophytes.

As defined above, 463, 492, 451, 471 and 464 OTUs were picked out as the core OTUs of cultivar Alexis, Barke, Marthe, Salome, and Simba respectively. 404 core OTUs were found present in all the five cultivars of barley seed endophytes (Figure 3-1-5a).

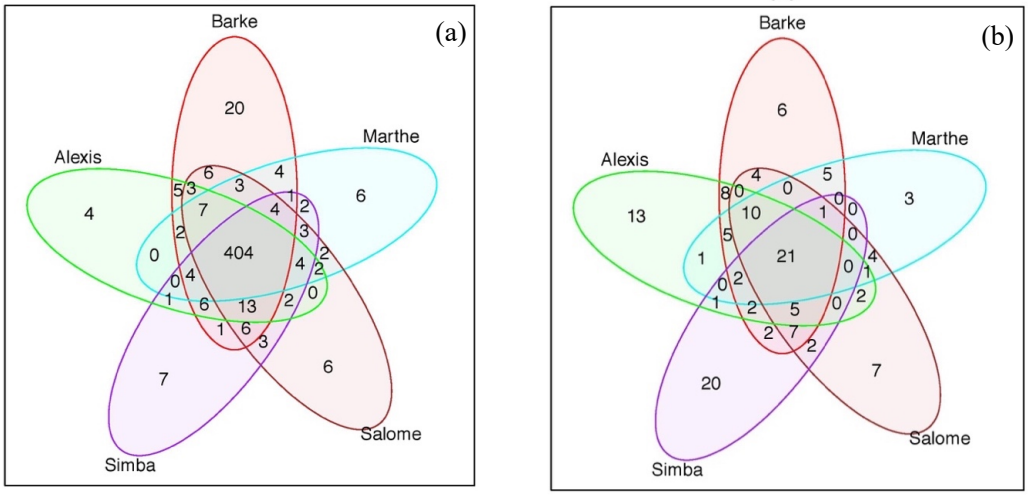


Figure 3-1-5 Core OTUs of (a) the resident groups and (b) potentially active groups of barley bacterial seed endophytes based on 16S rDNA and 16S rRNA amplicon sequencing respectively

The taxonomy of the core OTUs was in accordance with the major groups in seed endophytes (Supplementary Table S1). More than half of the core OTUs (214 OTUs) were assigned to

Enterobacteriaceae, accounting for more than 75% of the total reads. Within the family Enterobacteriaceae, 125 OTUs were assigned to *Enterobacter*, 8 OTUs to *Pantoea*, 5 OTUs to *Erwinia*, 1 OTU to *Trabulsiella* and 1 OTU to *Xenorhabdus.* Besides Enterobacteriaceae, there were also several other dominant groups. 98 OTUs were assigned to Pseudomonadaceae, comprising around 10% the total reads. 44 OTUs were assigned to Paenibacillaceae and 18 OTUs to Xanthomonadaceae. The remaining OTUs were all low in abundance (Supplementary Table S1).

3.1.1.2 Potentially active groups

137 genera from 83 families of 10 different phyla were detected in barley seeds using 16S rRNA based sequencing. Proteobacteria, Firmicutes, and Actinobacteria dominated the potentially active seed endophytic community. The most abundant families were Phyllobacteriaceae, Enterobacteriaceae, Paenibacillaceae, Microbacteriaceae, and Pseudomonadaceae (Figure 3-1-6).

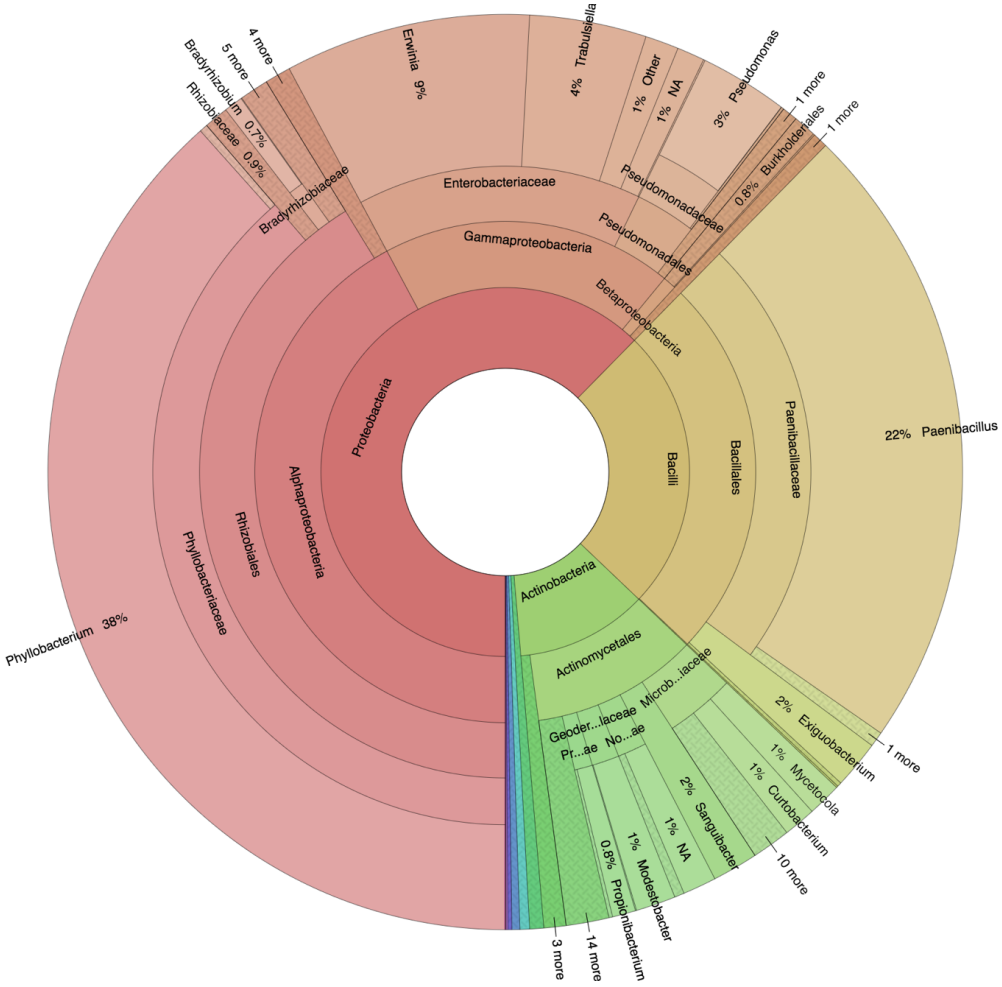


Figure 3-1-6 The taxonomic structure of the potentially active barley seed endophytes based on 16S rRNA amplicon sequencing (n=25). The Krona radial space-filling chart shows the mean relative abundances of bacterial taxa in seeds.

At the genus level, the highly abundant groups belonged to *Phyllobacterium*, *Paenibacillus*, *Erwinia*, *Traubulsiella*, and *Pseudomonas*. More than 70% of the total reads on average were assigned to these genera (Figure 3-1-6). Notably, *Paenibacillus* showed much higher abundance in cultivar Marthe than in other cultivars (Figure 3-1-7).

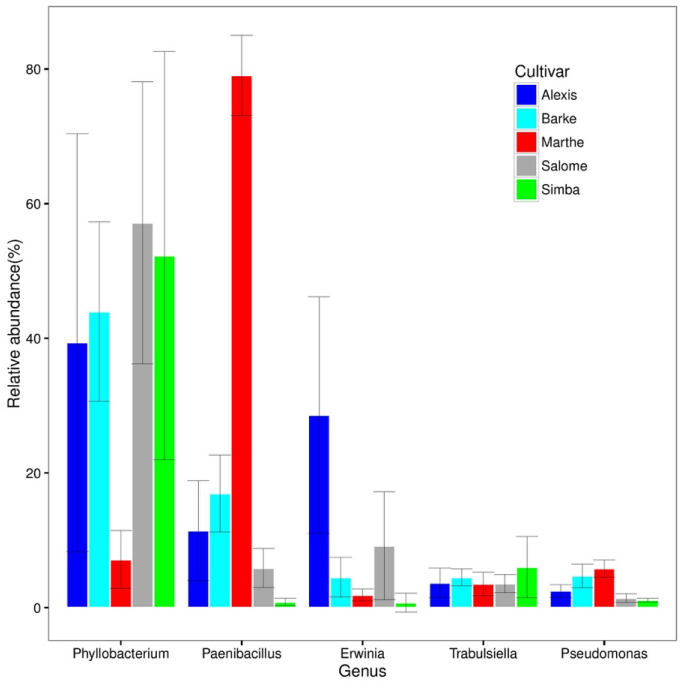


Figure 3-1-7 The most abundant genera of potentially active seed endophytes in different barley cultivars based on 16S rRNA amplicon sequencing (n=5)

We investigated the cultivar effects in the potentially active seed endophytes. Alpha diversity across the cultivars did not show statistical differences (Figure 3-1-8). However, differences in the bacterial community across cultivars were revealed by permutational multivariate analysis using Unifrac distance metrics. Cultivar Marthe was clearly separated from others in the PCoA plot based on weighted Unifrac (Figure 3-1-9 (A)).

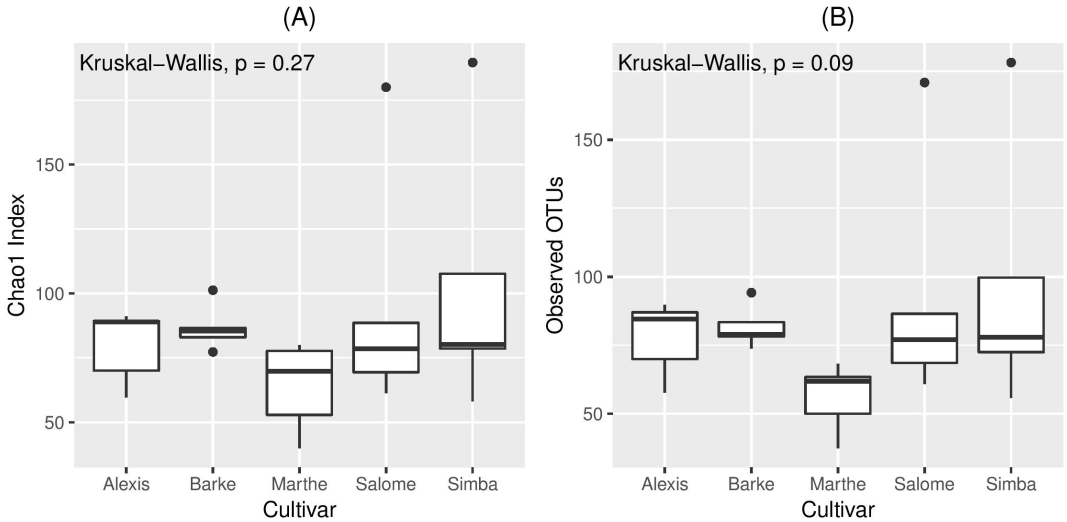


Figure 3-1-8 Alpha diversity (A) Chao 1 index and (B) observed OTUs of potentially active endophytes in five cultivar of barley seeds (n=5)

Using the method described above, 21 core OTUs were identified in barley seeds (Figure 3-1-5b). Among them, 4 OTUs were assigned to Phyllobacteriaceae, 5 OTUs to Paenibacillaceae, 5 OTUs to Enterobacteriaceae and 3 OTUs to Pseudomonadaceae (Supplementary Table S2). The rest 4 OTUs were assigned to Oxalobacteraceae (*Ralstonia*), Comamonadaceae (*Delftia*), Xanthomonadaceae (*Stenotrophomonas*) and Propionibacteriaceae (*Propionibacterium)* respectively (Supplementary Table S2). In total, these core OTUs represented 69.56% of all reads on average. Notably, the core OTUs in high abundance were assigned to *Phyllobacterium*, *Paenibacillus*, *Pseudomonas*, and *Trabulsiella*, all of which belonged to the dominant groups in the potentially active community of seed endophytes.

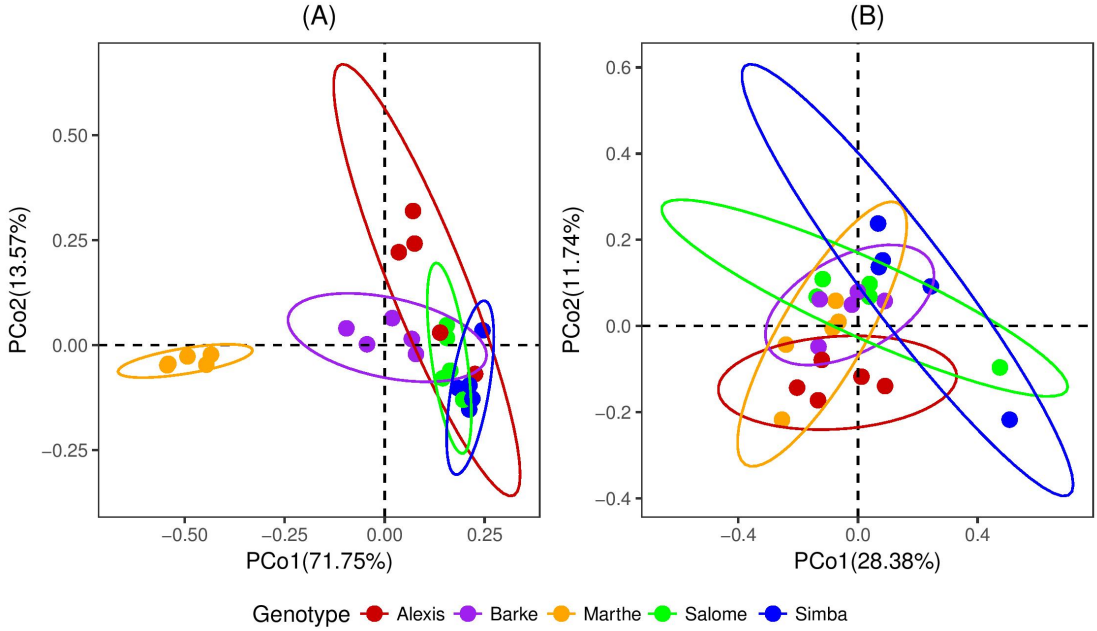


Figure 3-1-9 PCoA plot of potentially active seed endophytes in five barley cultivars based on (A) weighted (B) unweighted Unifac distances (n=5)

3.1.2 Seed-borne endophytes vertically transmitted to barley roots in axenic systems

3.1.2.1 Resident groups

In the resident root endophytes of barley grown in axenic systems, three phyla were detected using DNA based sequencing, namely Proteobacteria, Firmicutes and Actinobacteria (Figure 3-1-10). Particularly, Actinobacteria was in extremely low abundance, accounted for only 0.2% of total reads on average. The major families in the resident root endophytes from axenic systems were the same as the groups found in resident seed endophytes, which are Enterobacteriaceae, Paenibacillaceae, and Pseudomonadaceae (Figure 3-1-10). A similar pattern was also observed at the genus level. In the root endophytes, the major genera fell into *Enterobacter*, *Pseudomonas*, and *Paenibacillus* (Figure 3-1-11).

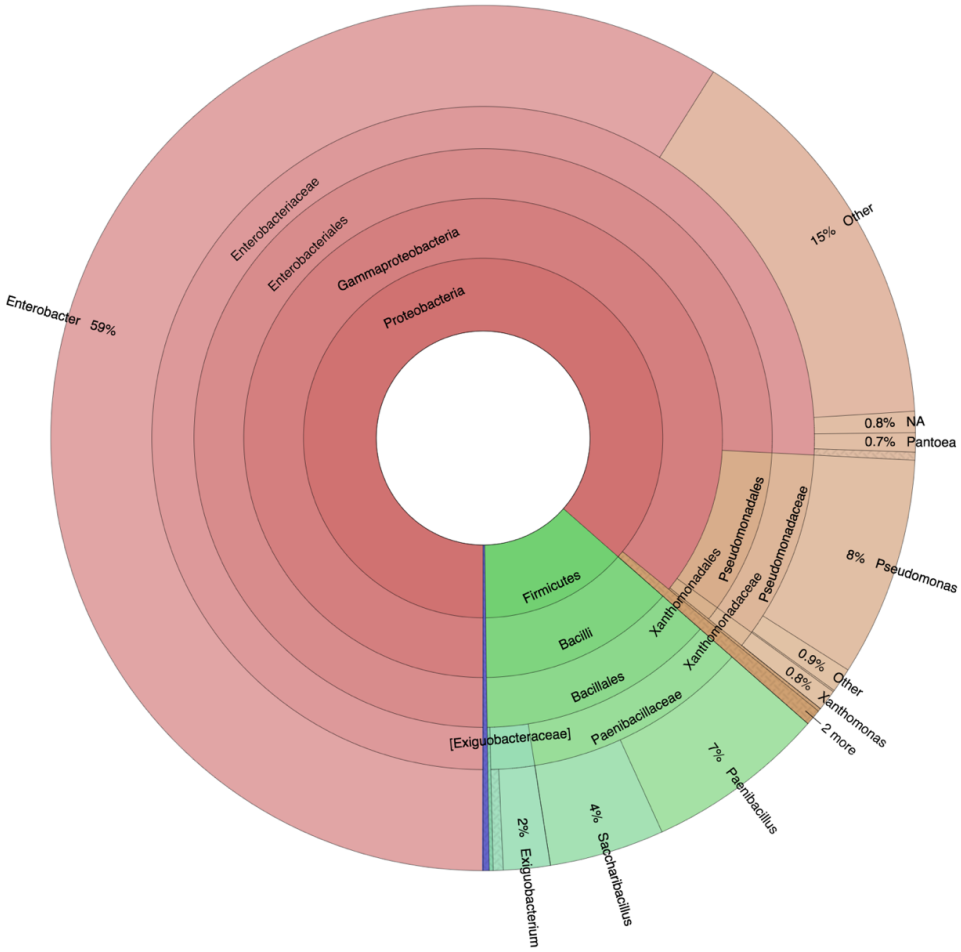


Figure 3-1-10 The taxonomic composition of resident groups of root endophytes in axenic systems based on 16S rRNA gene (16S rDNA) amplicon sequencing (n=25). The Krona radial space-filling chart shows the mean relative abundances of bacterial taxa in roots grown in axenic systems.

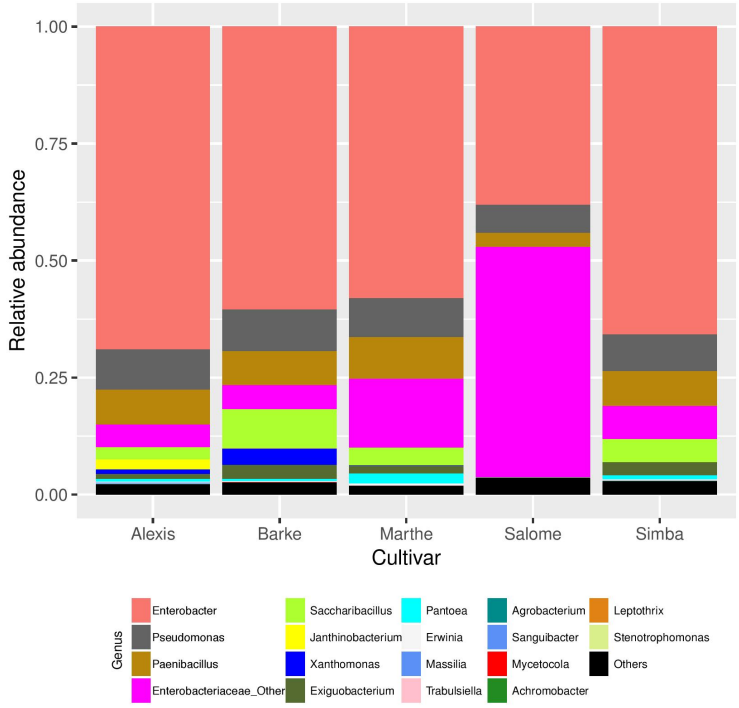


Figure 3-1-11 The major genera of resident root endophytes from five barley cultivars grown in axenic systems based on 16S rRNA gene (16S rDNA) amplicon sequencing (n=5)

Comparing the Chao1 index and observed OTUs, the alpha diversity across cultivars differed significantly in the resident bacterial root endophytes (Figure 3-1-12). Differences in ß diversity were also detected using permutational multivariate analysis. Although statistical tests with both weighted and unweighted Unifrac metrics were significant ($p<0.05$), the clustering pattern was observed only in the PCoA plot with unweighted Unifrac, where cultivar Salome was clearly separated from other cultivars (Figure 3-1-13).

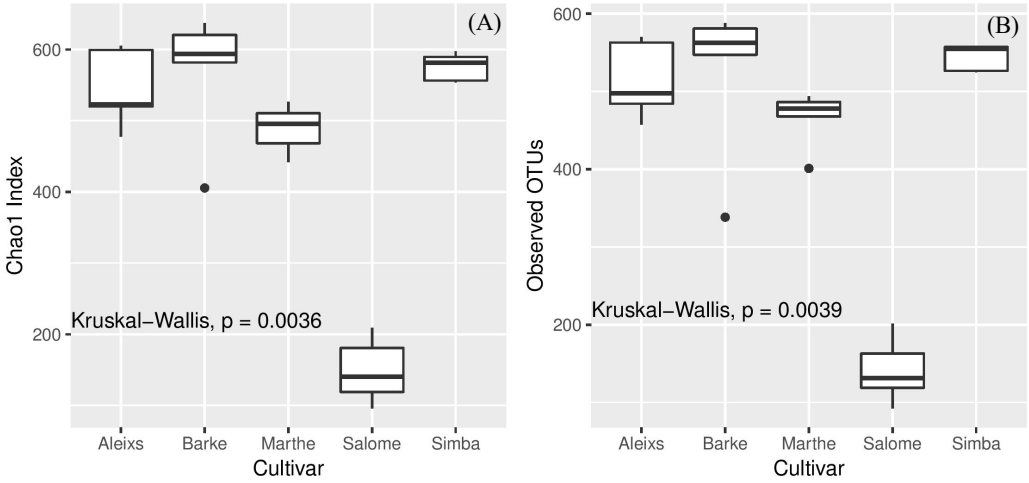


Figure 3-1-12 Alpha diversity (A) Chao 1 index and (B) observed OTUs of resident root endophytes from five barley cultivars growing in axenic systems (n=5)

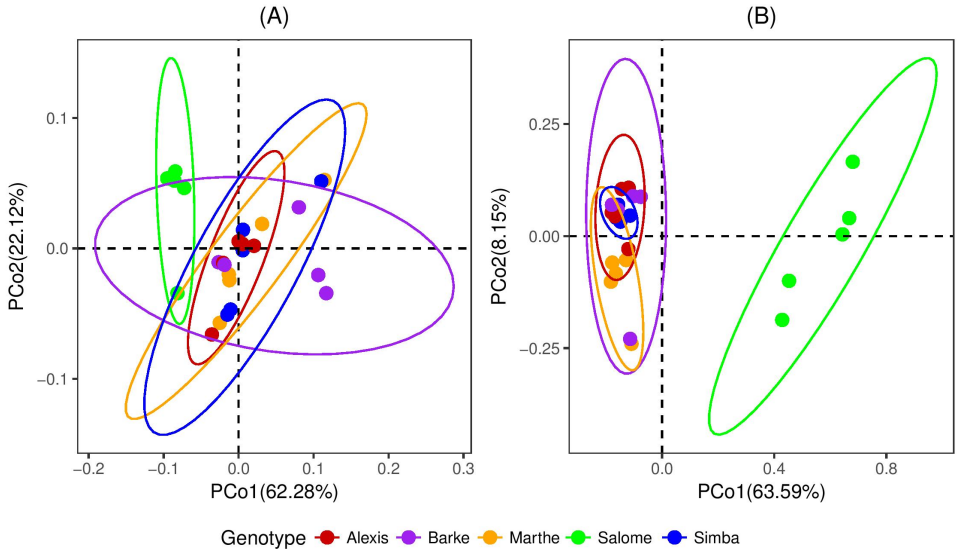


Figure 3-1-13 PCoA plot of resident root endophytes from five barley cultivars growing in axenic systems based on (A) weighted (B) unweighted Unifac distances (n=5)

In the resident root endophytes, 39 core OTUs were found in all investigated cultivars (Figure 3-1-14a). Taxonomically, all the core OTUs were in accordance with the major families in the whole community of root endophytes (Supplementary Table S3). 28 OTUs were assigned to Enterobacteriaceae, including *Enterobacter*, *Erwinia*, and *Trabulsiella*. Eight OTUs were assigned to *Pseudomonas* and one OTU to *Paenibacillus*.

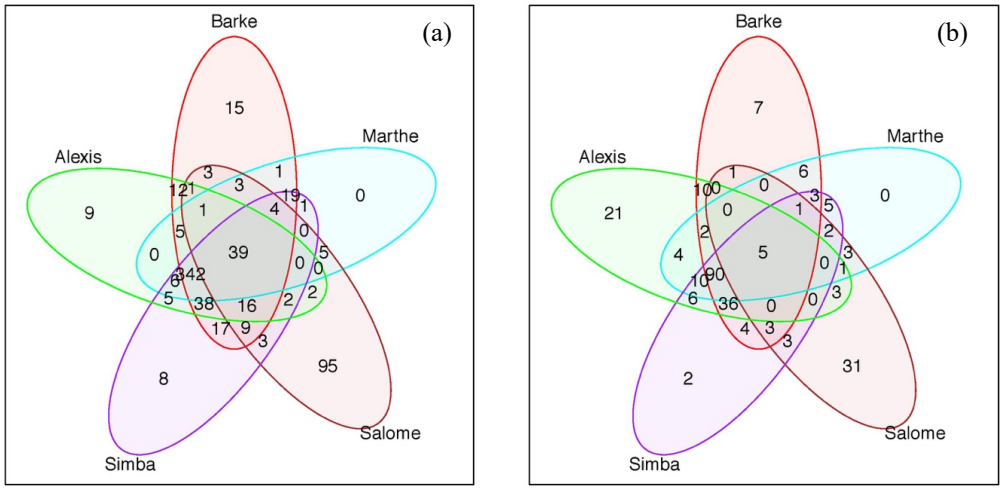


Figure 3-1-14 Core OTUs of (a) the resident and (b) potentially active groups of bacterial endophytes in roots of barley in axenic systems based on 16S rDNA and 16S rRNA amplicon sequencing respectively

To detect the seed-borne endophytes in roots from axenic systems, we compared the seed and root microbiome. Taking all the OTUs into consideration, 651 OTUs were found in both seeds and roots in the resident groups based on DNA sequencing, accounting for 98.50% of the total reads in seeds and 98.26% in roots (Figure 3-1-15). Taxonomically, all the genera recovered in roots were also detected in the seeds as expected. On the contrary, a few genera were only found in seeds but not observed in roots, including *Propionibacterium*, *Nitrospira*, *Burkholderia* and *Diapharobacter*.

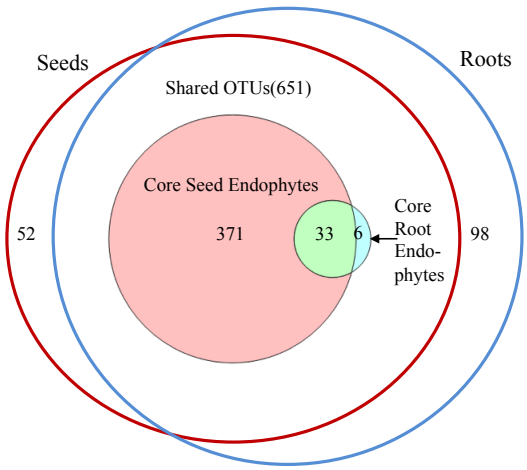


Figure 3-1-15 Overlaps of OTUs in the resident groups of seed and root endophytes based on 16S rRNA gene sequencing. The red circle represents the seed microbiome while the blue circle represents the root microbiome.

A large overlap was found in the core OTUs of barley seeds and roots. Thirty-three core OTUs were shared by seed and root endophytes (Figure 3-1-15). The shared core OTUs were mainly assigned to *Enterobacter*, *Pseudomonas*, *Erwinia*, *Trabulsiella*, and *Paenibacillus*. Notably, the abundance of a few OTUs assigned to *Enterobacter* (OTU1205, OTU2379, OTU552376 and OTU7904) was largely increased in roots compared with those in seeds (Supplementary Table S4). In total, the shared core OTUs represented 23.34% of the total

reads on average in the seed microbiome, while they accounted for 49.02% in the resident groups of root endophytes.

3.1.2.2 Potentially active groups

Three phyla, 11 families and 22 genera were found in the potentially active bacterial endophytes of roots in axenic systems. The major groups in roots were similar as those found in seeds, except for Phyllobacteriaceae, which dominated in seeds but was almost negligible in roots (Figure 3-1-16). The most abundant families were Enterobacteriaceae, Paenibacillaceae, Pseudomonadaceae, and Microbacteriaceae, all of which were also highly abundant in seed microbiome (Figure 3-1-16).

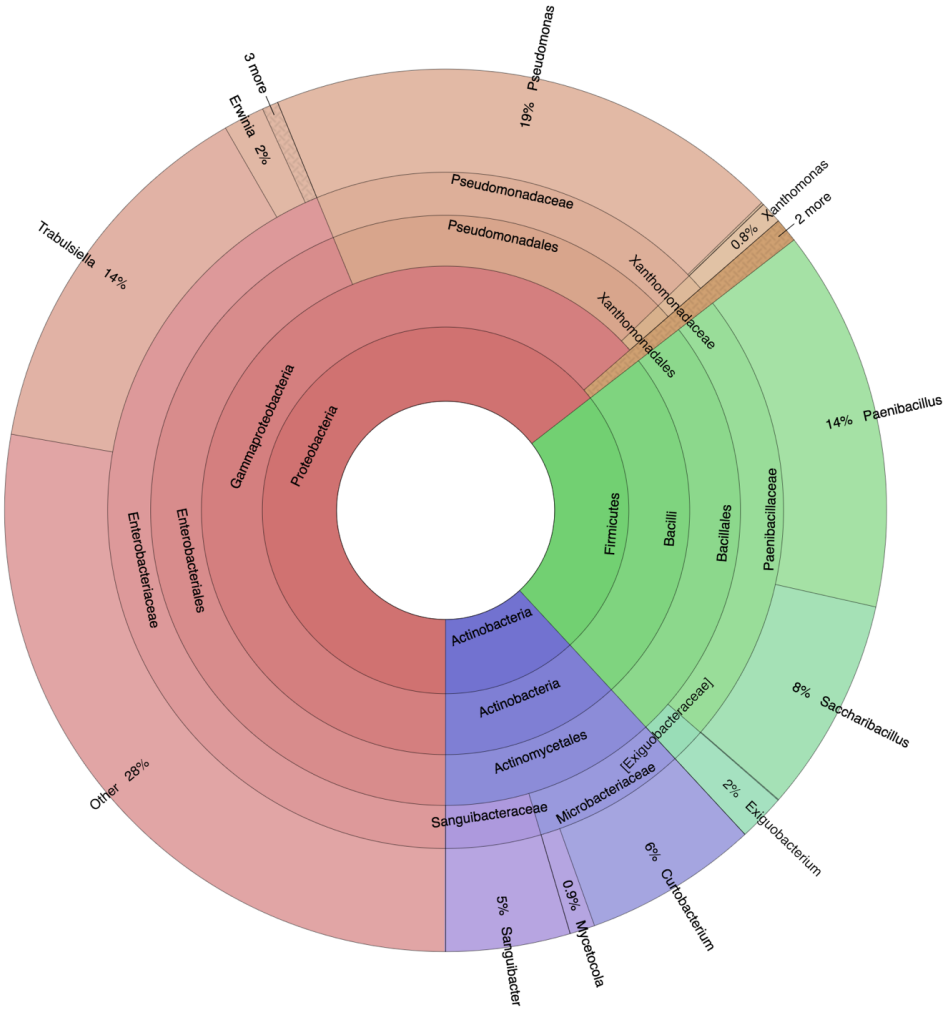


Figure 3-1-16 The taxonomic composition of potentially active groups of root endophytes in axenic systems based on 16S rRNA amplicon sequencing (n=25). The Krona radial space-filling chart shows the mean relative abundances of bacterial taxa in roots grown in axenic systems.

At the genus level, *Phyllobacterium*, the dominant group in seeds, was detected in roots but only with a mean relative abundance of less than 0.1%. Instead, the most abundant group belonged to Enterobacteriaceae, although with an ambiguous classification at the genus level (appeared as 'other') (Figure 3-1-17). Other genera in Enterobacteriaceae, like *Erwinia*,

Enterobacter, and *Pantoea* were also detected but with much lower abundance. The major genera were found to be *Pseudomonas*, *Paenibacillus*, *Trabulsiella*, *Saccharibacillus, Curtobacterium*, and *Sanguibacter*. Notably, *Trabulsiella* showed much higher abundance in cultivar Salome than in other cultivars (Figure 3-1-17).

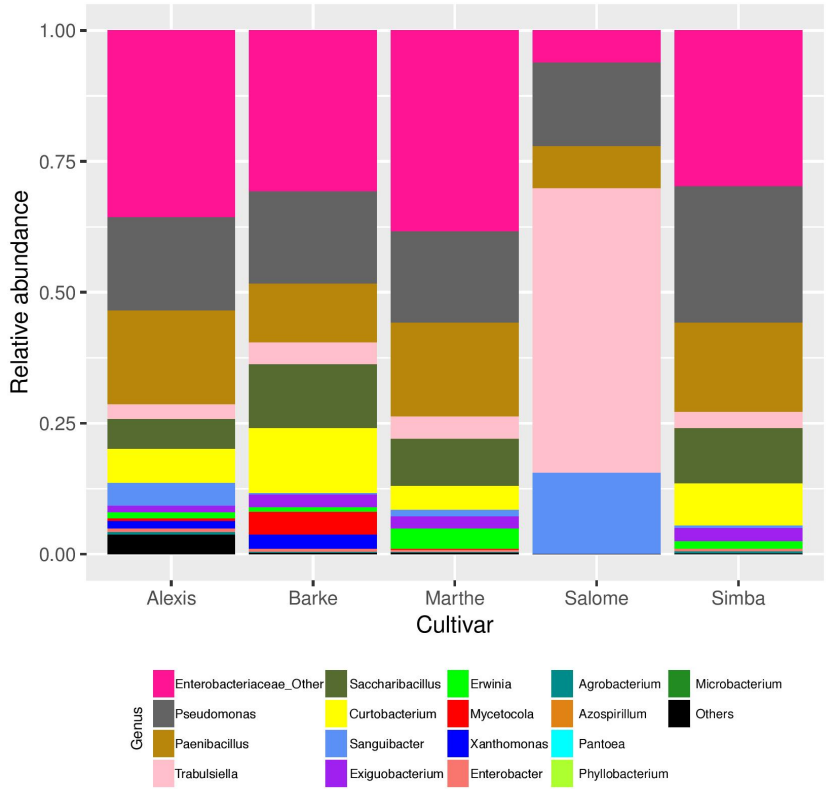


Figure 3-1-17 The major genera of potentially active root endophytes from five barley cultivars growing in axenic systems based on 16S rRNA gene amplicon sequencing (n=5)

As like in the resident groups, alpha diversity was found differ across cultivars in the potentiallt active root endophytes (Figure 3-1-18). Similarly, the lowest alpha diversity was also observed in cultivar Salome.

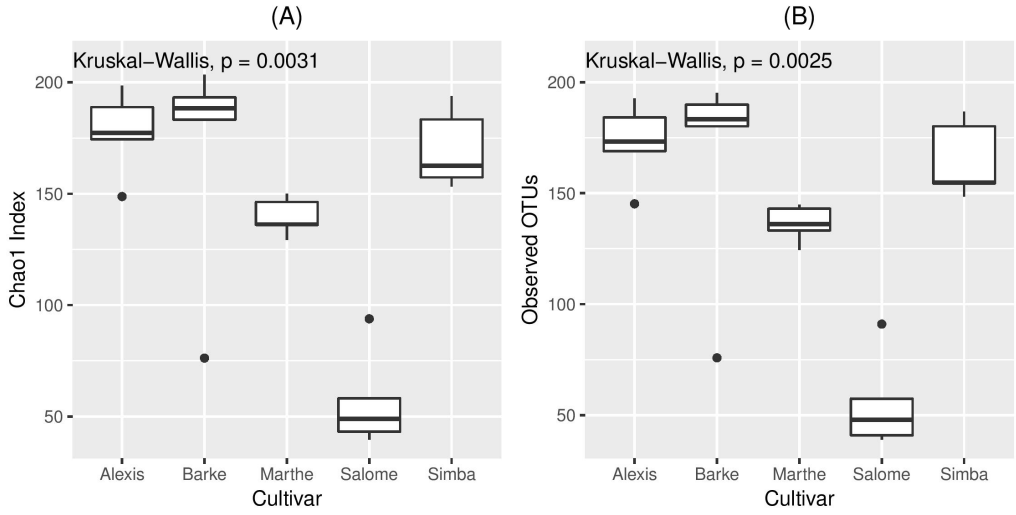


Figure 3-1-18 Alpha diversity (A) Chao 1 index (B) observed OTUs
of active root endophytes from five barley cultivars growing in axenic systems (n=5)

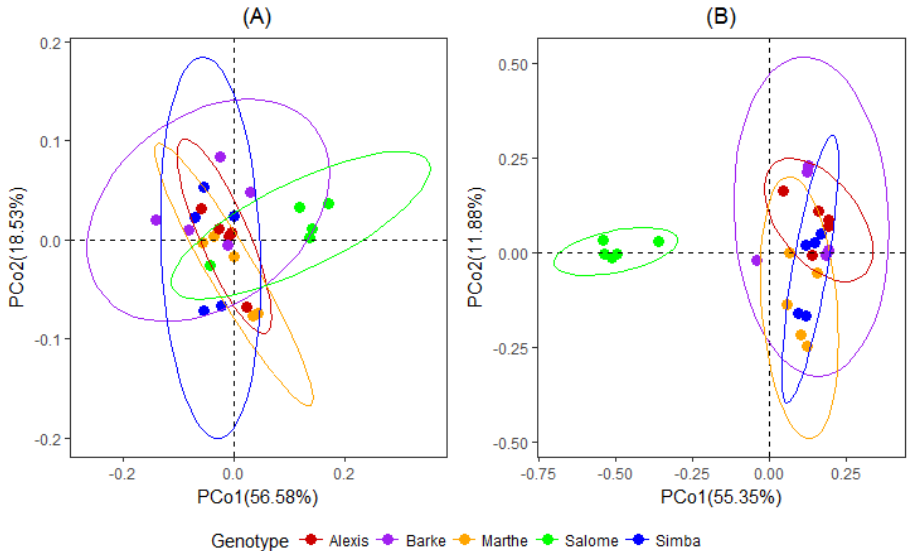


Figure 3-1-19 PCoA plot of active root endophytes from five barley cultivars growing in axenic systems based on (A) weighted (B) unweighted Unifac metrics (n=5)

Cultivar effects were significant using both weighted and unweighted Unifrac distance metrics (Figures 3-1-19). The clustering pattern could be clearly observed in the PCoA plot with the unweighted Unifac, where Salome was clearly separated from others (Figure 3-1-19).

In the potentially active endophytes of roots grown in axenic systems, only five core OTUs were shared by all the cultivars (Figure 3-1-14b), which were assigned to the family Enterobacteriaceae and Pseudomonadaceae. Interestingly, they were also the most abundant families in the seed and root associated microbiome. Two OTUs (OTU791973 and OTU725048) were highly abundant among the core OTUs but varied largely in the abundance across cultivars. They represented more than 35% total reads in all the cultivars except Salome, where they only accounted for less than 0.1% of total reads (Supplementary Table S5).

Four core OTUs were found in both seed and root endophytes (Figure 3-1-20), which were assigned to *Trabulsiella* and *Pseudomonas*. All the four core OTUs were largely increased in the relative abundance after seeds were developed into roots (Supplementary Table S6). The shared core OTUs altogether represented 4.39% of the total reads in the potentially active seed microbiome and 38.60% total reads in root microbiome.

In the entire active endophytic community, 185 OTUs were shared in seed and root endophytes, accounting for 83.31% of the total reads in seeds and 84.46% in roots respectively (Figure 3-1-20). Taxonomically, more assigned genera were detected in the seeds. Seventy-five genera from 49 families in the active seed endophytes were not found in the active groups of root endophytes. Most of these missing genera were in low abundance in seeds (less than 0.5%). On the contrary, all genera recovered from roots were retrieved from

seeds, except for one genus *Xanthomonas*, which was only detected in the roots but not in seeds. Comparing the composition of active seed and root endophytes, the most noteworthy difference was *Phyllobacterium*. Being the most dominant genus in active seed endophytes, *Phyllobacterium* was negligible in roots.

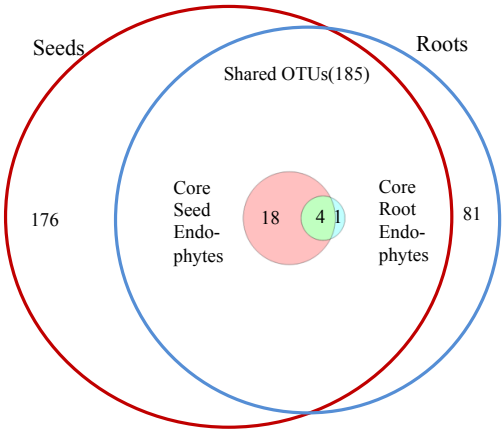


Figure 3-1-20 Overlaps of OTUs in the potentially active groups of seed and root endophytes based on 16S rRNA sequencing. The red circle represents the seed microbiome while the blue circle represents the root microbiome

3.1.3 Seed-borne endophytes became less abundant in barley roots grown in soil

As expected, the composition of root endophytes from barley grown in soil was much more complex than that of plants from axenic systems. Based on 16S rRNA sequencing, 165 genera from 97 families of 12 phyla were detected at the seedling stage, while 201 genera from 101 families of nine phyla were found at the booting stage. In comparison, the active root endophytes from axenic systems only covered three phyla, 11 families and 22 genera, all of which were also observed in barley grown in soil. Proteobacteria, Firmicutes, and Actinobacteria remained to be the dominant phyla in roots of barley grown in soil. Particularly, a much higher abundance of Actinobacteria was found in roots grown in soil than in axenic systems (Figure 3-1-21).


Seedling Stage
Proteobacteria
Actinobacteria
Firmicutes
Alphaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Actinomycetales
Thermoleophilia
Bacilli
Rhizobiales
Xanthomonadales
Pseudomonadales
Enterobacteriaceae
Pseudomonadaceae
Xanthomonadaceae
Sinobacteraceae
Rhizobiaceae
Bradyrhizobiaceae
Phyllob...riaceae
Rhodospirillaceae
Sphingomonadaceae
Oxalobacteraceae
Comamonadaceae
Streptomycetaceae
Micrococcaceae
Micromonosporaceae
Frankiaceae
Noca...ceae
Clostridiales
Clostridiaceae
Bacillales
Paenibacillaceae
Sphin...dales
Rhodosp...ilales
Caulob...raceae
Burkho...riales
Soli...rales
Pseudomonas 4%
Erwinia 1%
Other 23%
Agrobacterium 1%
Other 0.7%
Rhizobium 0.6%
Bradyrhizobium 2%
Phyllobacterium 1%
2 more
Methylobacterium 0.7%
4 more
Azospirillum 7%
Sphingomonas 1%
Other 0.9%
1% Janthinobacterium
5 more
1% Other
11 more
6% Streptomyces
0.9% Other
6% Other
2% Other
0.7% Cryocola
3 more
5% Other
3% Other
2% Mycobacterium
2% Rhodococcus
15 more
8% Other
2% Paenibacillus

(A)

(B)

Figure 3-1-21 The taxonomic structure of root endophytes from barley plant grown in soil. Krona radial space-filling charts showed the mean relative abundances of bacterial taxa at
(A) seedling stage and (B) booting stage detected by 16S rRNA amplicon sequencing.

At the seedling stage of barley grown in soil, the most abundant family in roots was Enterobacteriaceae (Figure 3-1-21 (A)), the same as barley grown in axenic systems. Family Microbacteriaceae was found in high abundance in root endophytes in both systems. Other highly abundant groups in roots grown in soil were Clostridiaceae, Streptomycetaceae, Rhodospirillaceae, and Actinosynnemataceae. However, these families were not detected or in negligible percentage in in roots in axenic systems. Paenibacillaceae and

Pseudomonadaceae, which were dominant in the axenic systems, were also detected in roots grown in soil, though in much lower abundance (Figure 3-1-21 (A)).

At booting stage, Pseudomonadaceae turned out to be the most abundant family. Rhizobiaceae, Streptomycetaceae, Microbacteriaceae, Bacillaceae, Enterobacteriaceae, Nocardiaceae and Comamonadaceae were also among the major groups (Figure 3-1-21 (B)).

At the genus level, the most abundant genera were assigned to *Pseudomonas*, *Agrobacterium*, *Streptomyces*, *Bacillus*, *Rhodococcus* and *Microbacterium* (Figure 3-1-21 (B)).

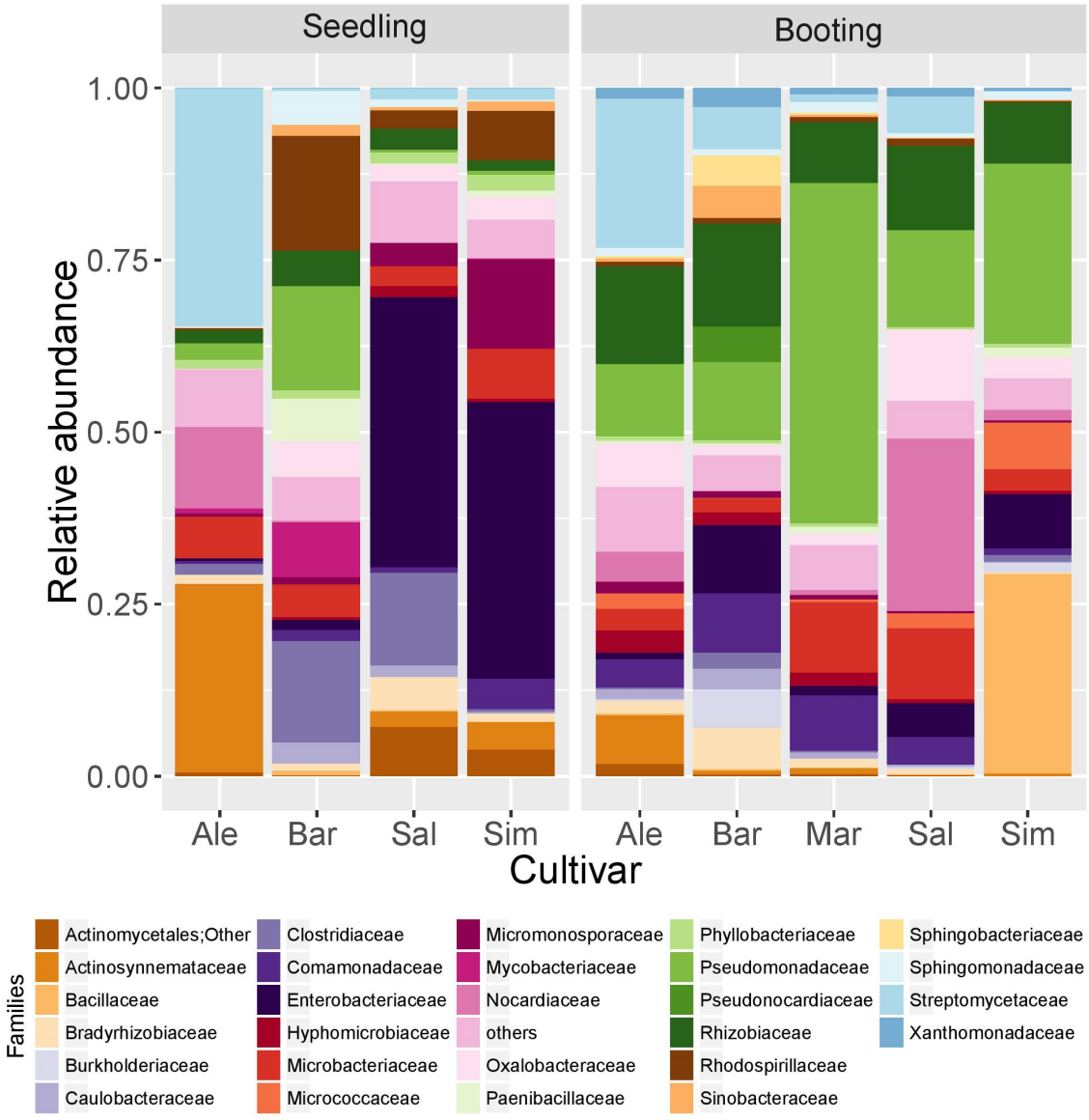


Figure 3-1-22 The major families in root endophytes of different barley cultivars grown in soil in the greenhouse at seedling and booting stage detected by 16S rRNA sequencing (n=3-4)

Comparing to the root endophytes in axenic systems, much larger variability among cultivars was observed in the taxonomic composition of root endophytes in soil. (Figure 3-1-22).

We compared the Chao1 index and observed OTUs of root endophytes in each cultivar at two growth stages separately (Figure 3-1-23). No statistical differences in alpha diversity across cultivars were detected, neither at seedling or booting stage.

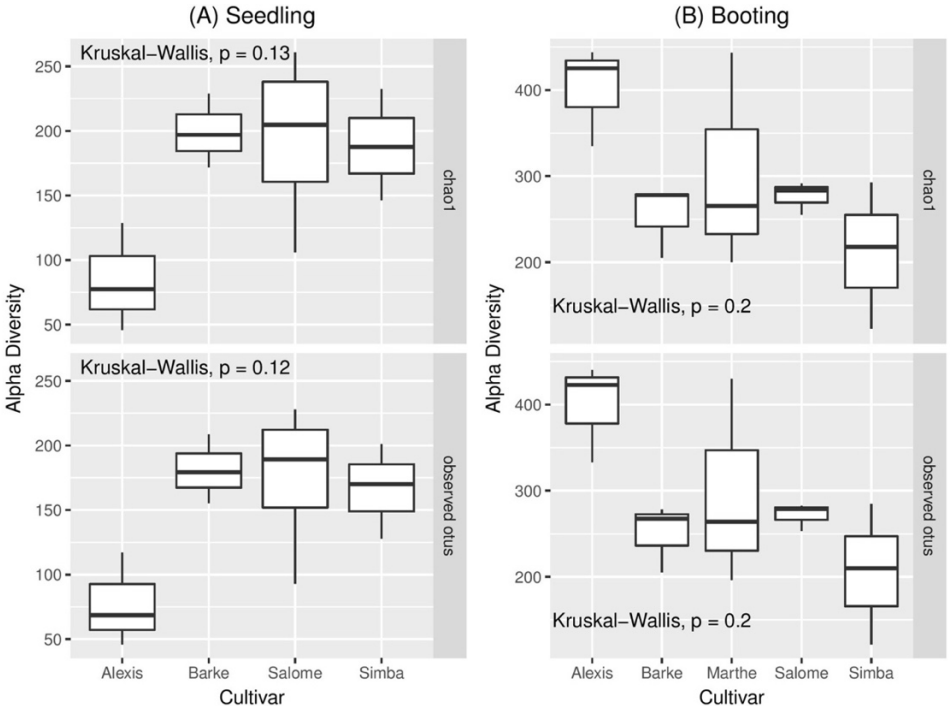


Figure 3-1-23 Alpha diversity of root endophytes from different barley cultivars grown in soil at (A) seedling and (B) booting stage (n=3-4)

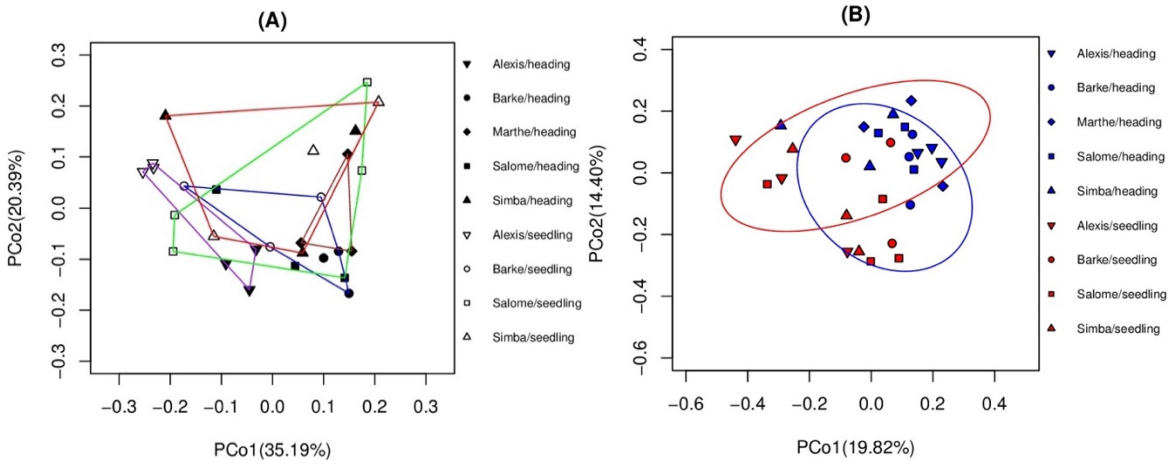


Figure 3-1-24 PCoA plots of bacterial endophytes of roots from different barley cultivars grown in soil harvested at seedling and booting stage based on (A) weighted and (B) unweighted Unifrac distance metrics

Statistical analysis using weighted Unifrac distances revealed the cultivar dependent impacts on barley root endophytes (Figure 3-1-24A). However, the cultivar effects were not significant using unweighted Unifrac metrics (Figure 3-1-24B). The results indicated that the cultivar dependent effects were mainly due to the difference in taxonomic relative abundance rather than the presence/absence of specific OTUs.

To gain insights into the growth stage effects, we compared the alpha diversity of the root endophytes at seedling and booting stage (Figure 3-1-25). The Chao1 index and observed OTUs were found higher at booting stage than at seedling stage (t-test, $p < 0.05$), suggesting higher alpha diversity at the booting stage.

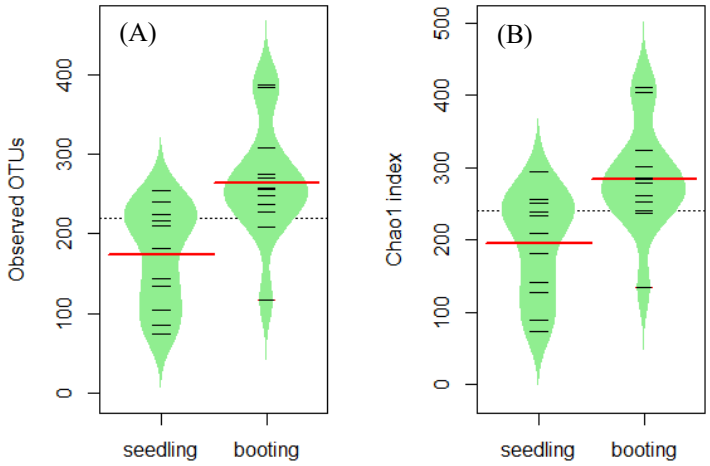


Figure 3-1-25 (A) Observed OTUs and (B) Chao1 index of bacterial endophytes in the roots of barley plants grown in soil during seedling and booting. Each sample is represented by a black horizontal line. The green area represents the estimation of the distribution. Red line indicates the average level (n=12-15)

We further carried out permutational multivariate analysis. The results indicated that growth stages accounted for the variation between microbial communities using both weighted and unweighted Unifrac distance. However, the clustering pattern by growth stages was only visible in the ordination plot based on unweighted Unifrac (Figure 3-1-24). Both the alpha and beta diversity implied that the plants' developmental stage is the main driving factor in shaping the root associated bacterial community.

At seedling stage, 19 OTUs were shared by all the cultivars as the core OTUs of the potentially active endophytes (Figure 3-1-26a), spanning three phyla, seven orders, 13 families and 18 genera (Supplementary Table S7). Noteworthy, most of the core OTUs were assigned to Actinomycetales, Rhizobiales and Burkholderiales. In general, the core OTUs were in low abundance of less than 1%. They were not among the most abundant groups in the whole community of root endophytes at seedling stage as well. Altogether, the 19 core OTUs accounted for 11.81% of the total reads on average.

At booting stage, 60 OTUs were identified as the core OTUs (Figure 3-1-26b), covering two phyla, four classes, nine orders, 19 families and 34 genera (Supplementary Table S8). As in the seedling stage, the core OTUs were found mainly belonged to Actinomycetales, Rhizobiales, and Burkholderiales. In contrast to the seedling stage, the taxa of the core OTUs

at booting stage were among the most abundant groups in root endophytes, e.g. *Pseudomonas*, *Agrobacterium*, and *Microbacterium*. Altogether, the 60 core OTUs represented 46.45% of the total reads on average.

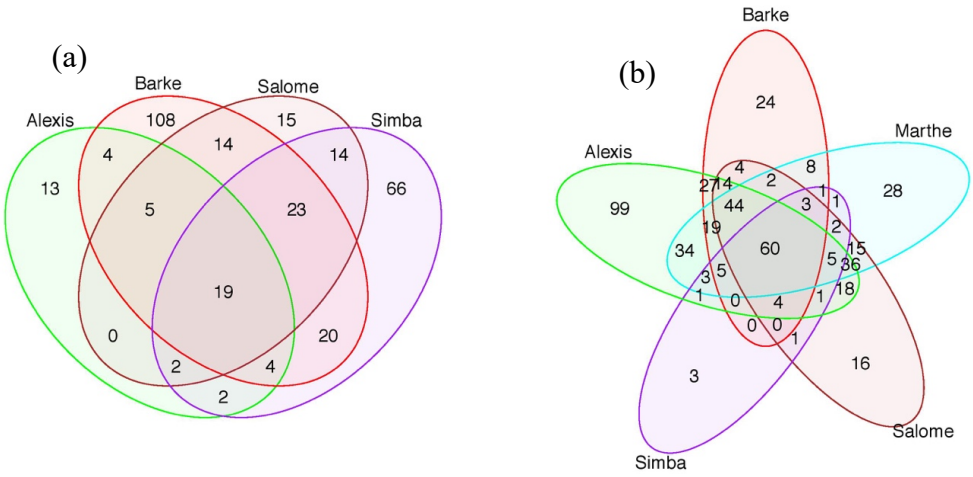


Figure 3-1-26 Core OTUs of each cultivar and all the five cultivars in the active bacterial endophytes roots in soil at (a) seedling stage and (b) booting stage based on 16S rRNA amplicon sequencing

Although there was a large overlap between the OTUs at two growth stages (574 OTUs), only 12 core OTUs were shared across all cultivars in both seedling and booting stages in the root endophytes (Figure 3-1-27). Not surprisingly, the core OTUs mainly belong to the order of Actinomycetales, Rhizobiales and Burkholderiales. At family level, the core OTUs were assigned to Bradyrhizobiaceae, Rhizobiaceae, Phyllobacteriaceae, Comamonadaceae, Actinosynnemataceae, Propionibacteriaceae, Caulobacteraceae and Sphingomonadaceae (Supplementary Table S9).

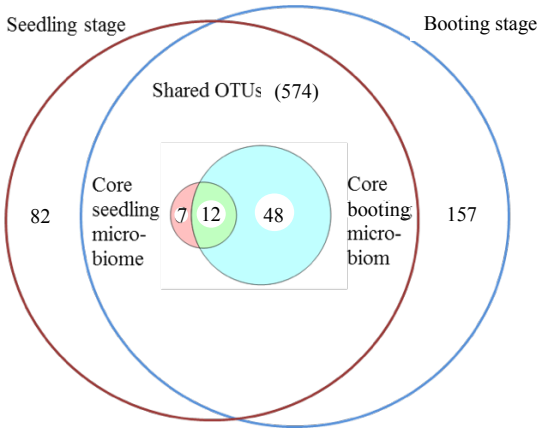


Figure 3-1-27 Shared OTUs of active root endophytes at seedling and booting stage based on 16S rRNA amplicon sequencing. The red circle represents root microbiome at seedling stage and the blue circle represents the root microbiome at booting stage.

To identify the seed-borne endophytes in roots, we further compared seed and root microbiome. Overall, a large set of OTUs were shared by seeds and roots grown in soil (Figure 3-1-28). More than half of the OTUs present in seeds (274 OTUs out of 475 OTUs

accounting for 59.70% of the total reads) were recovered from roots at both seedling and booting stages, indicating that a great part of root endophytes may originate from the seed microbiome.

At seedling stage in soil, 344 more OTUs were detected in roots, while 407 more were found when plants were further developed to booting stage. As expected, the percentage of the shared OTUs decreased during the development of barley plants. The shared OTUs represented 37.56% and 27.51% of the total reads at seedling and booting stage respectively. Apparently, roots at two growth stages shared much more OTUs than they shared with seeds.

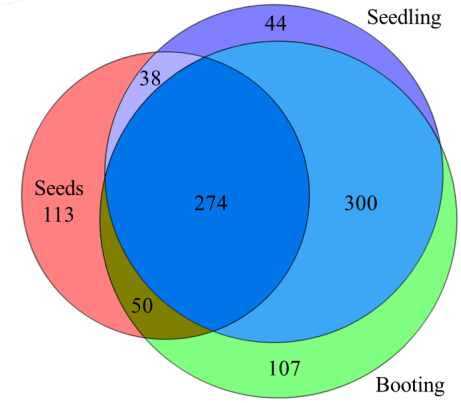


Figure 3-1-28 Overlaps of OTUs in the active seed and root endophytes at both seedling and booting stages based on 16S rRNA sequencing

Most of the core OTUs in seeds belonged to the abundant groups in the root endophytes. However, few of the core OTU in roots at the seedling stage represented the most abundant genera. After barley plants developed from seedling to booting stage, the core OTUs were found comprised mainly of the dominant groups in the community again.

Four core OTUs were shared in seeds and roots at seedling stage, which were assigned to Propionibacteriaceae, Phyllobacteriaceae and Oxalobacteraceae (Supplementary Table S10). Notably, OTU219107, which was assigned to *Phyllobacterium*, dropped drastically from 37.99% in seeds to 0.87% in roots. Similarly, *Phyllobacterium*, the most abundant genus in the active seed endophytes, decreased to less than 1% in seedlings.

For barley plants at booting stage, five core OTUs were shared by seeds and roots, which were assigned to Propionibacteriaceae, Phyllobacteriaceae, Pseudomonadaceae, and Xanthomonadaceae (Supplementary Table S11). Most of the OTUs were low in abundance, except two OTUs (OTU791973 and OTU578606) assigned to *Pseudomonas*. Two core OTUs (OTU165421 and OTU705063) were found in seed and root endophytes at both growth stages (Supplementary Table S12), indicating their stable presence and activity in barley seeds and roots. OTU165421, which was assigned to *Propionibacterium*, decreased in the relative abundance from seeds to roots and during the development of plants. The other core

OTU705063, which was assigned to *Mesorhizobium*, was in relatively comparable abundance in seeds and roots.

We further compared the taxonomy in the composition of the potentially active endophytic community in seeds and roots. Roots at two growth stages were treated as a whole. In total, we found 73 taxa differed in the relative abundance in seeds and roots (Supplementary Table S13). Among them, 59 taxa were enriched in roots grown in soil (Kruskal-Wallis test, Bonferroni corrected p value < 0.05), most of which belonged to Actinomycetales, Rhizobiales, and Burkholderiales.

Particularly, 21 genera were not found in seeds at all but appeared in roots, indicating their soil-origin. Two representative genera were *Streptomyces* and *Clostridium*, which were not detected in seeds but were in high abundance (7.84% and 3.61% respectively) in roots. The rest 38 genera were in low abundance in seeds but were significantly increased in roots grown in soil.

Fourteen taxa showed a decrease of relative abundance in roots compared to seeds (Kruskal-Wallis test, Bonferroni corrected p value < 0.05). The two most prominent genera were *Phyllobacterium* and *Paenibacillus*, both of which were dominating in seeds. However, in roots grown in soil, they dropped from 39.30% and 23% respectively to less than 1%.

3.2 Functional potentials of barley seed-borne endophytes in roots

3.2.1 The taxonomic composition of seed-borne endophytes in barley roots

In this study, DNA was extracted from the bacterial pellets. Metagenomic library was constructed and sequenced on Miseq platform. In total, 17,484,440 paired-end raw reads were obtained. The number of reads per sample varied from 634,594 to 6,386,752. After adaptor removal and PhiX contamination clean up, 17,483,850 high-quality paired-end reads were left for taxonomy and function analysis. A high coverage of the metagenome sequencing was indicated by the nonpareil curves (Figure3-2-1), which are 0.80±0.13 and 0.87±0.05 for Barke and Salome respectively.

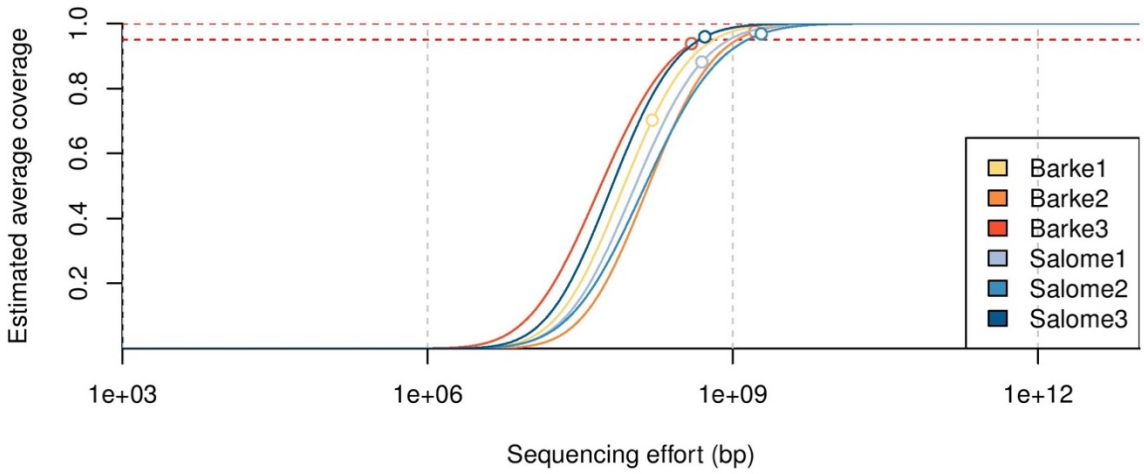


Figure 3-2-1 Nonpareil curves for the metagenomes of bacterial root endophytes in barley cultivar Barke and Salome. The horizontal dashed lines indicate 100% (orange) and 95% (red) coverage. The empty circles indicate the size and estimated coverage of the datasets. The lines after the points are projections of the fitted model.

We did the taxonomic classification for the metagenome sequencing with the classifier Kaiju (Menzel et al. 2016). Reads were directly assigned using the NCBI taxonomy and a reference database of protein sequences from microbial and viral genomes built by Kaiju. With this direct assignment, we found that more than 89% of the reads were classified (Figure 3-2-2). Sequences assigned to bacteria accounted for around 87% to 95% of the raw reads in each sample (Figure 3-2-2). Besides bacteria, a few reads were also assigned to archaea, viruses, and eukaryotes including fungi.

In the classified reads, the percentage of the bacterial sequences was more than 98% (Figure 3-2-3). Plant-derived sequences were detected under the superkingdom of Eukaryota, which was classified as ‘Viridiplantae’. However, the presence of plant-derived sequences was almost negligible, with a percentage of 0.076% ± 0.053%.

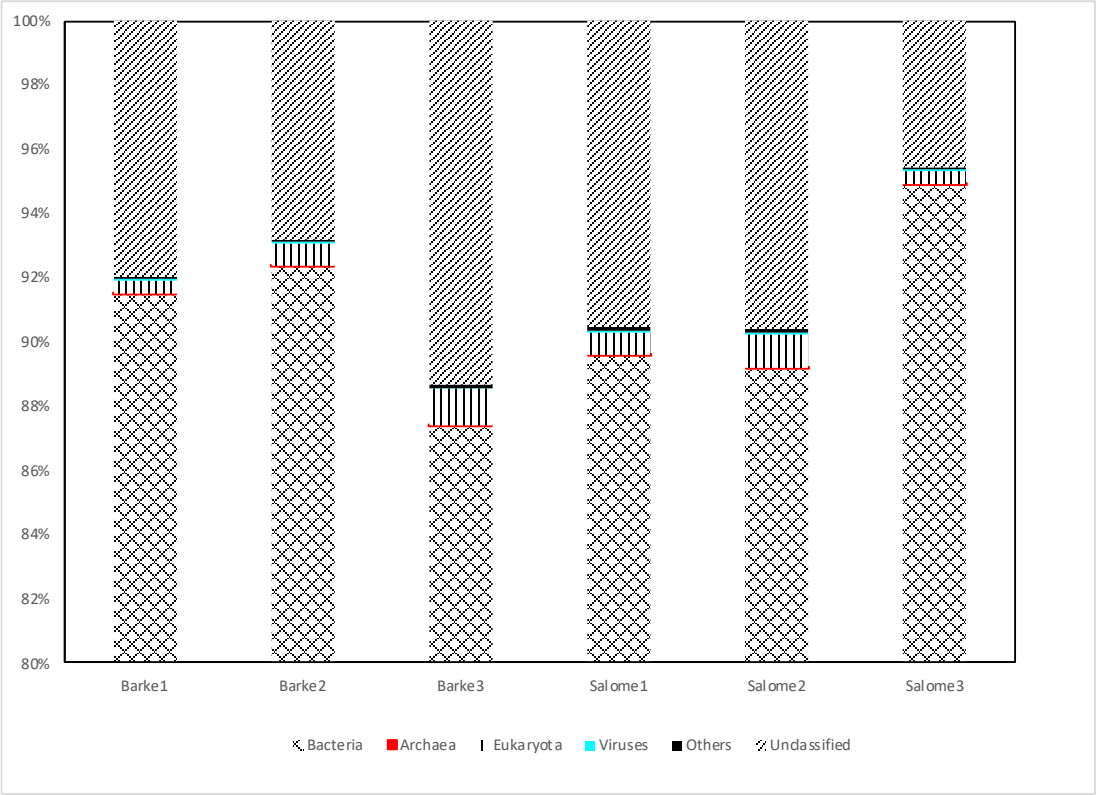


Figure 3-2-2 The taxonomic structure assigned by Kaiju in the raw reads of each sample. Unclassified reads represent less than 10% of the raw reads in each sample.

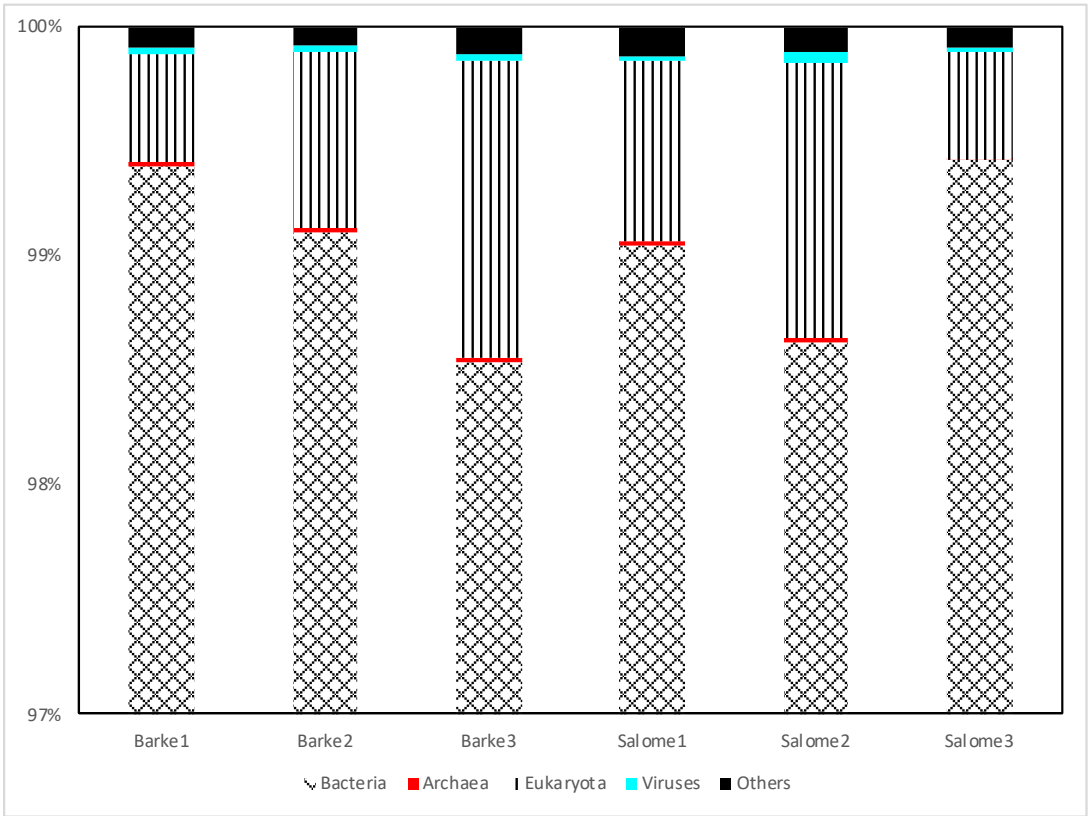


Figure 3-2-3 The relative abundance and taxonomic composition of all groups in the classified reads assigned by Kaiju.

In the following analysis, we only focused on bacteria as our main target. The direct assignment showed that the bacteria community is highly complex. More than one thousand genera were identified. However, the vast majority was in extremely low abundance (less than 1%) (Figure 3-2-4). In contrast, around 90% of all the reads were assigned to 10 major genera, namely *Pantoea*, *Agrobacterium*/*Rhizobium*, *Erwinia*, *Stenotrophomonas*, *Pseudomonas*, *Paenibacillus*, *Enterobacter*, *Achromobacter, Paraburkholderia* and *Lactobacillus* (Figure 3-2-4). Among them, *Pantoea* was largely in dominance. The relative abundance of the major genera in Barley cultivar Barke and Salome were shown in Figure 3-2-5.

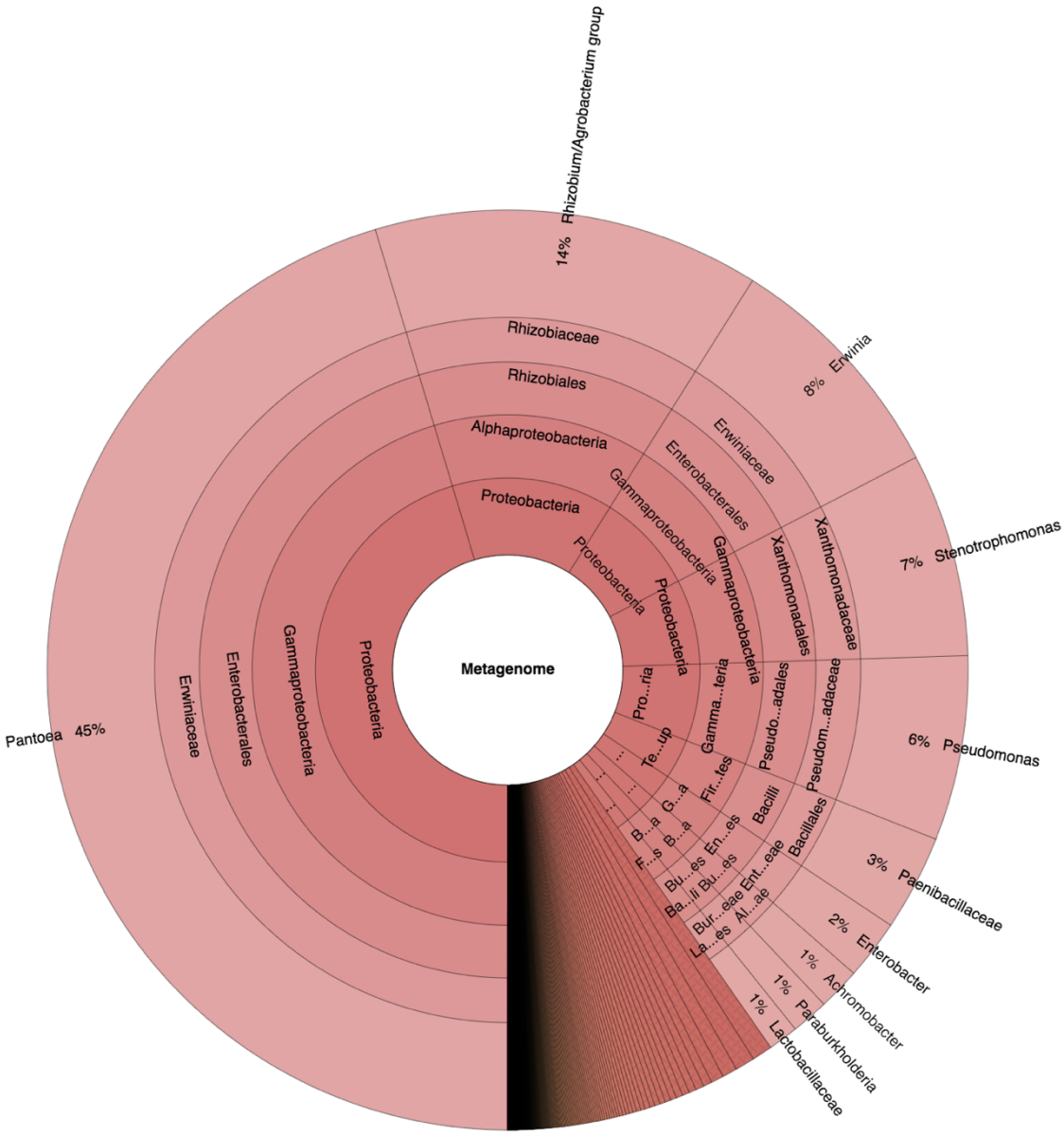


Figure 3-2-4 The taxonomic structure of bacterial root endophytes with seed origin using direct assignment of metagenome sequencing by Kaiju

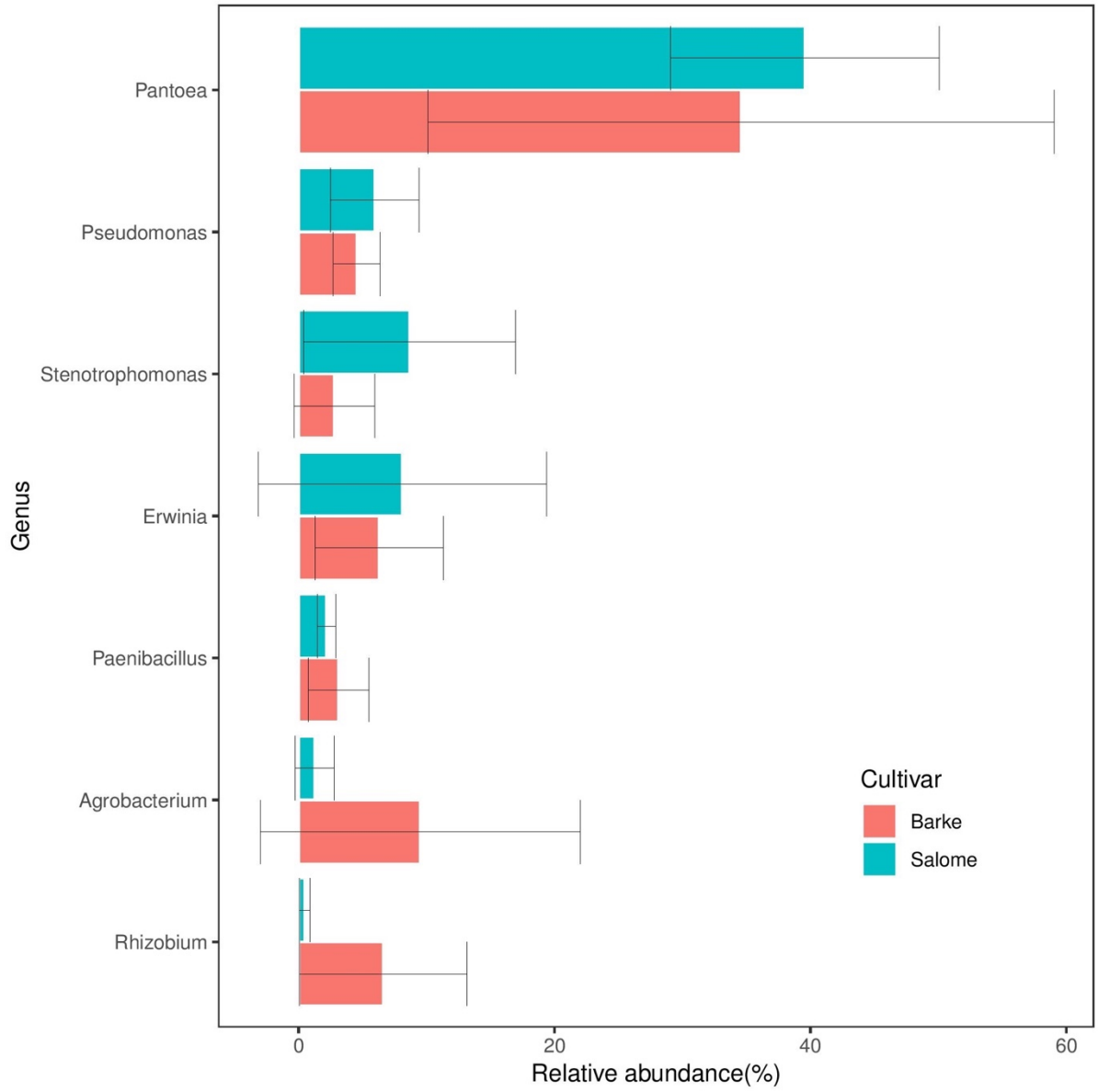


Figure 3-2-5 The relative abundance of the major genera in the bacterial root endophytes with seed origin in two barley cultivars detected in the metagenome sequencing by direct assignment with Kaiju

We further extracted the reads that belonged to the 16S ribosomal RNA (rRNA) gene and analyzed with Qiime pipeline. In total, 82,063 paired-end 16S rRNA reads were extracted, accounting for 0.5% of all the raw reads. 72,989 reads passed the quality filtering and were proceeded for the OTU calling. After removing singletons from the OTU map, 55,661 reads were left. Unclassified reads and reads assigned to archaea were discarded, resulting in 53,151 classified sequences. In the remaining sequences, 3,892 reads were identified as chloroplast or mitochondria and were hence filtered out. In the end, 49,259 bacterial 16S rRNA sequences were clustered into 684 OTUs.

Enterobacteriaceae was the most abundant family, with an average abundance of more than 50%. The major genera detected in the 16S rRNA reads were *Pantoea*, *Erwinia*,

Stenotrophomonas, *Pseudomonas*, *Agrobacterium*, *Paenibacillus*, *Burkholderia*, *Enterobacter* and *Achromobacter* (Figure 3-2-6). Most of the genera were in low abundance. Among all the 117 genera, 102 genera were below 1%. In general, the profile of the 16S rRNA reads was similar to the taxonomy detected by the direct assignment of metagenome sequencing.

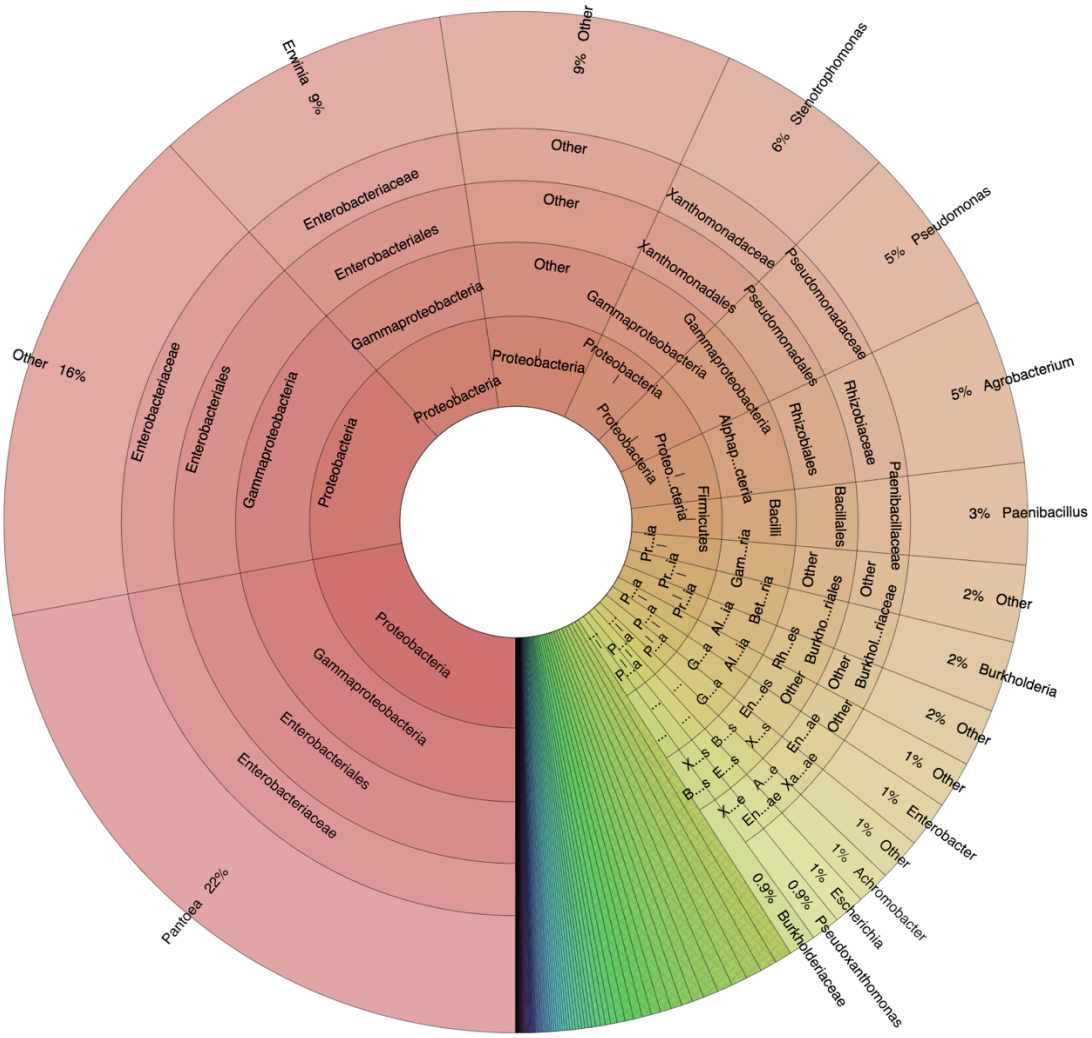


Figure 3-2-6 The structure of bacterial root endophytes with seed origin analyzed with Qiime pipeline using extracted16S ribosomal RNA (rRNA) gene from metagenome sequencing

3.2.2 Functional characteristics of root endophytes originated from seeds

To assess the functional capacities of the bacterial root endophytes originated from seeds, metagenomic reads were mapped to the database of Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000). Around 84% to 91% of the raw reads in each sample were annotated. A total of 13,117,378 sequences were assigned and classified with

specific KEGG metabolic pathways. In total, 4,383 KEGG orthologous group (KOs) were found.

To detect global differences within the functions of seed-borne root endophytes in two barley cultivars, we performed PCoA. Statistical analysis showed that there were no significant differences in functions between the two cultivars (Adonis, p>0.1) (Figure 3-2-7).

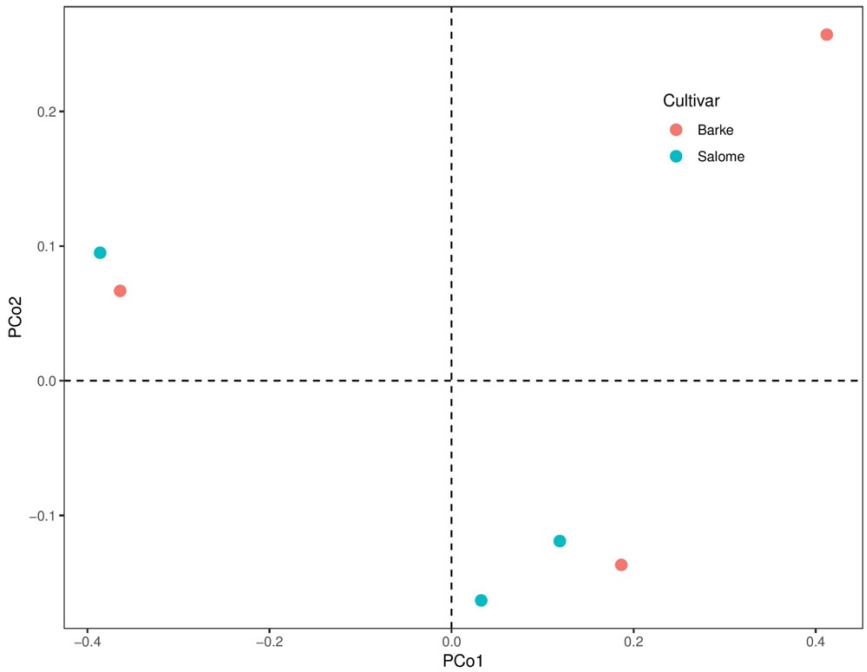


Figure 3-2-7 PCoA plot of functions of root endophytes in two barley cultivars with seed origin. The functions were annotated using KEGG (n=3).

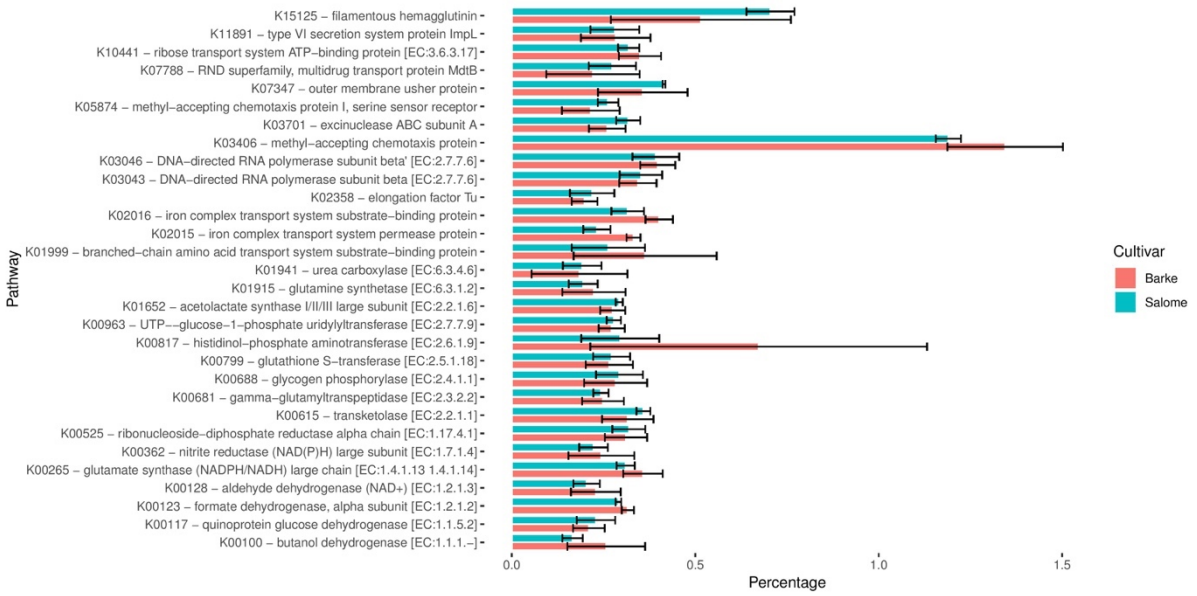


Figure 3-2-8 The top 30 most abundant functional pathways of seed-borne root endophytes in two barley cultivars.

We identified the top 30 most abundant KEGG Orthologs (KOs) in seed-borne root endophytes (Figure 3-2-8), including methyl-accepting chemotaxis protein, filamentous hemagglutinin, type VI secretion system protein ImpL, glutathione S-transferase, iron complex transport system and other essential functions for bacteria.
Besides the most abundant KOs, we further searched the functional traits expected in seed-borne endophytes, in the perspective of bacteria colonization and survival, effects on seed germination, and plant-microbe interaction.

Colonization and survival
Methyl-accepting chemotaxis protein (K03406) turned out to be the most abundant KO. The tsr-Methyl-accepting chemotaxis protein I (K05874) was also found among the top 30 most abundant KOs. In addition, all the pathways for bacterial chemotaxis were found in the seed-borne endophytes in barley roots, indicating chemotaxis is one of the key features in bacterial seed-borne endophytes.

Filamentous hemagglutinin (K15125) was the second most abundant functional pathway (Figure 3-2-8). Hemagglutinin is related to biofilm formation and adherence. Its high abundance suggests that bacterial endophytes require this function to successfully colonize plants.

Glutathione S-transferase (K00799) was also among the top 30 abundant KOs (Figure 3-2-8). Other pathways for antioxidant enzymes like catalase and superoxidase were found as well. Our results indicate that Reactive Oxygen Species (ROS) detoxification is needed for seed-borne endophytes to deal with oxidative stress.

The full pathways for osmoprotectant transport systems and glycine betaine/proline transport system were detected in our metagenome sequencing. The six pathways for the compatible solute trehalose biosynthesis were all detected, among which the maltose and maltooligosaccharides glycogen pathways were largely in dominance.

Nutrient acquisition
Starch is the most abundant reserve carbohydrate in barley seeds. Pathways for amylase were detected in the bacterial endophytes (Figure 3-2-9).
The hydrolysis of storage proteins is aided by proteinases. In the metagenome sequencing of bacterial endophytes, aminopeptidase was detected (Figure 3-2-9), which may potentially involve in the mobilization of reserve protein.

Besides, we also detected KOs for nitrogen fixation (*nifH*), however, with only 17 reads. Taxonomically, these reads belonged to *Paenibacillus* and *Raoultella* (formerly designated *Klebsiella*).

Phytate is the major storage form of phosphorus in seeds. As expected, we detected functional pathways of phytase in our metagenome sequencing (Figure 3-2-9).

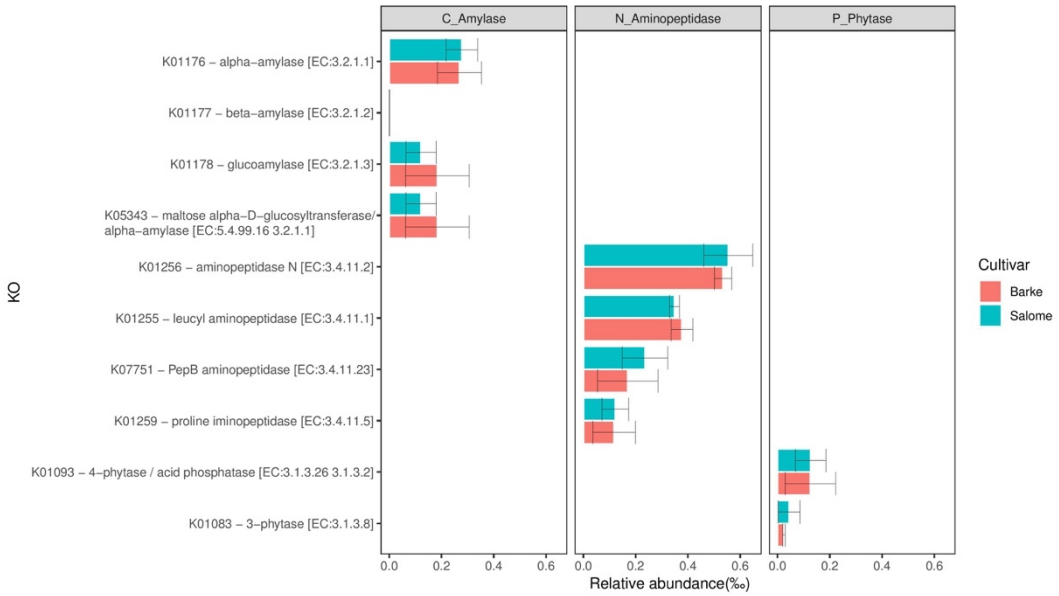


Figure 3-2-9 Functional pathways of nutrient mobilization and acquisition in seed-borne root endophytes, including amylase, aminopeptidase and phytase.

In the metagenome sequencing, pathways for siderophore biosynthesis were detected. The pathway of Bacillibactin and Yersiniabactin were complete. For Myxocheline, only the pathway for group A was complete. For Enterochelin, Pyochelin, Mycobactin, and Vibriobactin, the essential components of synthesis were missing (Figure 3-2-10).

Two KOs involved in iron transport, namely iron complex transport system substrate-binding protein (K02015) and iron complex transport system permease protein (K02016) were among the top 30 most abundant KOs (Figure 3-2-8). The full pathway of iron siderophore and mineral iron (III) transportation was also identified in our metagenomic annotation, suggesting a high potential of the barley bacterial endophytes to compete for iron in the plant endosphere.

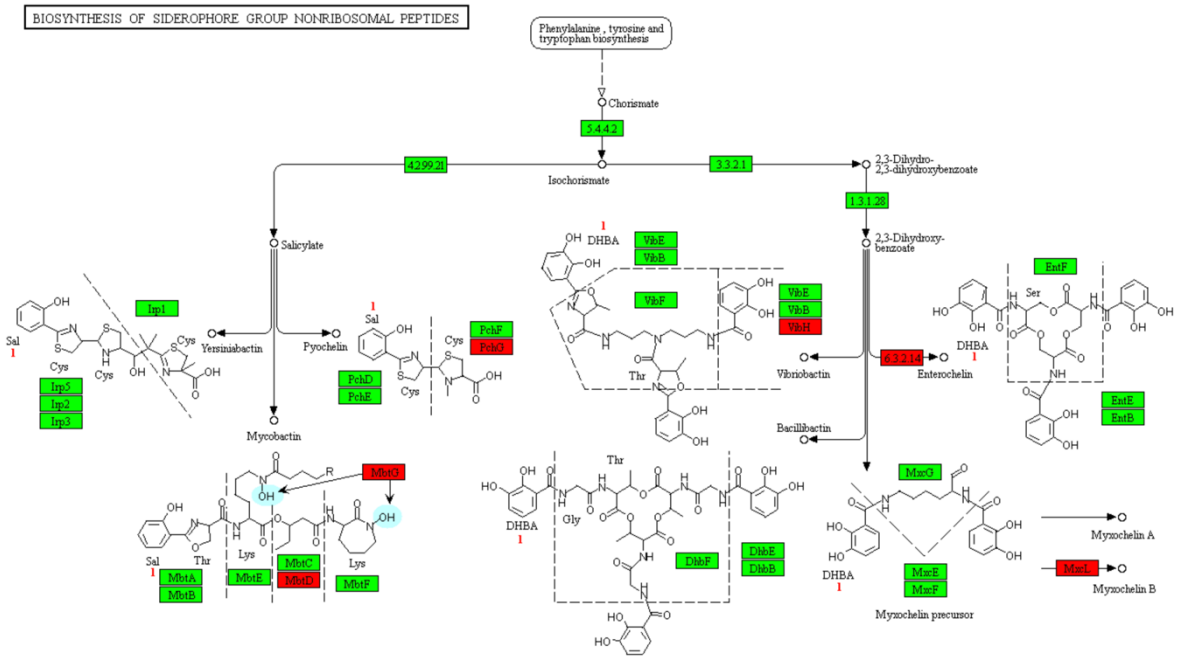


Figure 3-2-10 The KEGG pathway for siderophore biosynthesis in non-ribosomal peptides. The KOs detected in the metagenome sequencing were marked in green, while the missing KOs were marked in red.

Phytohormone production

Pathways for phytohormone production detected included ACC deaminase, auxin, and gibberellin.

Two pathways involving different intermediates and enzymes for IAA synthesis were identified, namely indole-3-acetamide (IAM) pathway and indole-3-pyruvate (IPA) pathway. The IPA pathway was more prevalent in the metagenome sequencing of seed-borne root endophytes, as the reads identified as IPA pathway was almost nine times that of the IAM pathway. Interestingly, the two pathways were found in distinct groups of bacteria. The IAM pathway mainly occurred in *Pseudomonas*. Only four reads in the IAM pathway were assigned to other bacteria. The IPA pathway was detected mainly in family Erwiniaceae, especially *Pantoea*, *Erwinia*, *Enterobacter*, and *Paenibacillus*. Generally, more reads were found for the IPA pathway, and bacteria with this pathway were also more diverse than that with the IAM pathway (Figure 3-2-11).

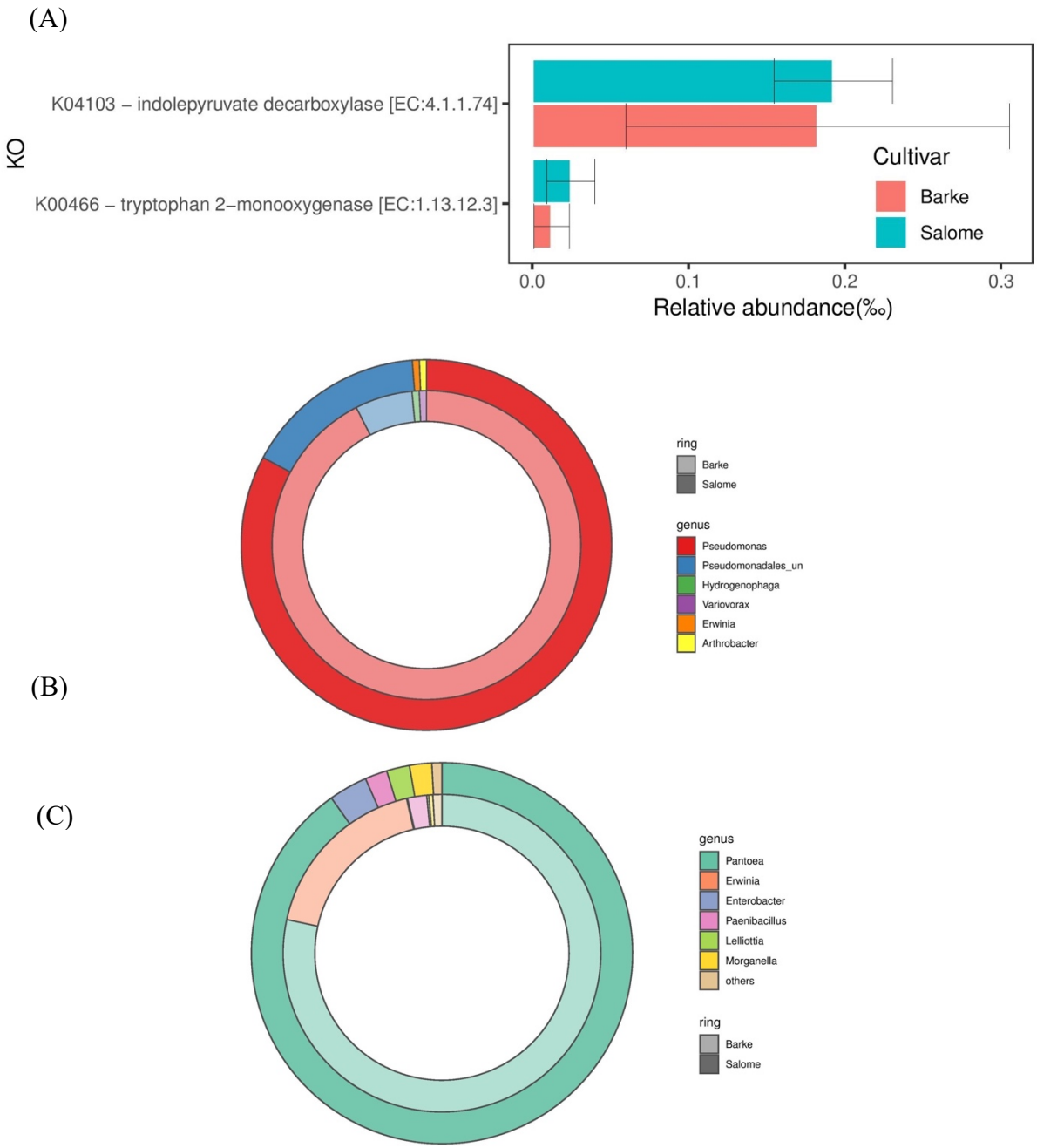


Figure 3-2-11 The bacterial endophytes in two barley cultivars (Barke and Salome) which harbor the (B) IAM and (C) IPA pathways of IAA synthesis. The relative abundance of the two pathways in each cultivar was shown in (A). The outer ring represents cultivar Salome, while the inner ring represents cultivar Barke. The colors in the inner ring are transparency modified colors of the outer ring.

We also detected pathways for gibberellin synthesis, including GA9, GA12, GA15, GA19, GA20, GA24, GA29, GA51, GA53 (Figure 3-2-12).

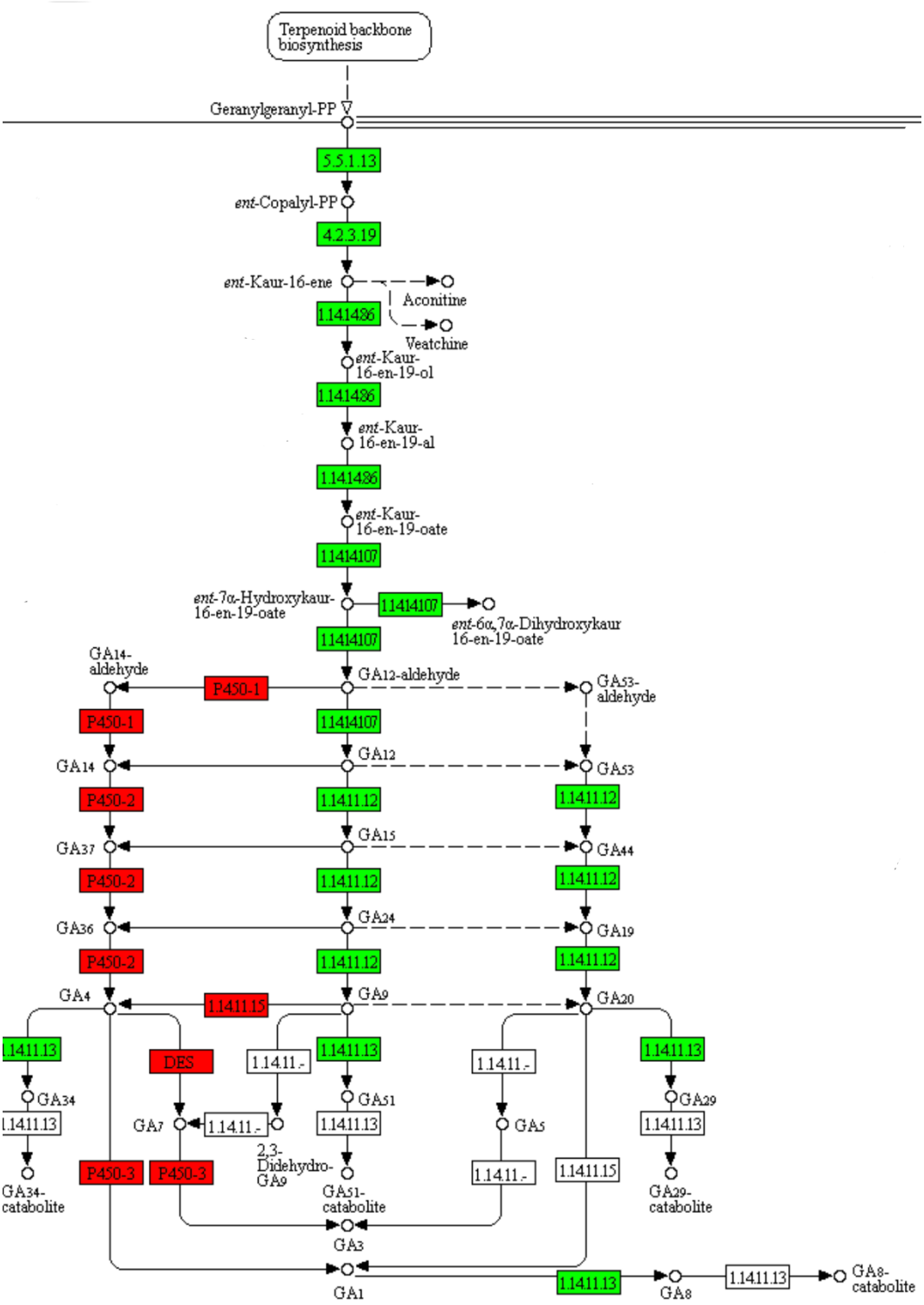


Figure 3-2-12 The KEGG pathway for gibberellin biosynthesis. The KOs detected in the metagenome sequencing were marked in green, while the missing KOs were marked in red.

Secretion systems

The Type VI secretion system (T6SS) appeared to be the most abundant secretion system in root endophytes, and type VI secretion system protein ImpL (K11891) was highly abundant in the metagenome (Figure 3-8). Complete pathways for type I secretion system (T1SS), type

IV secretion system (T4SS) and type VI secretion system (T6SS) were found. The presence of type II and type III secretion systems were also detected, however, with incomplete pathways. In type II secretion system (T2SS), the lipoprotein GspS was missing, while in type III secretion system (T3SS), the needle-forming component (YscO, YscP, YscX) was missing.

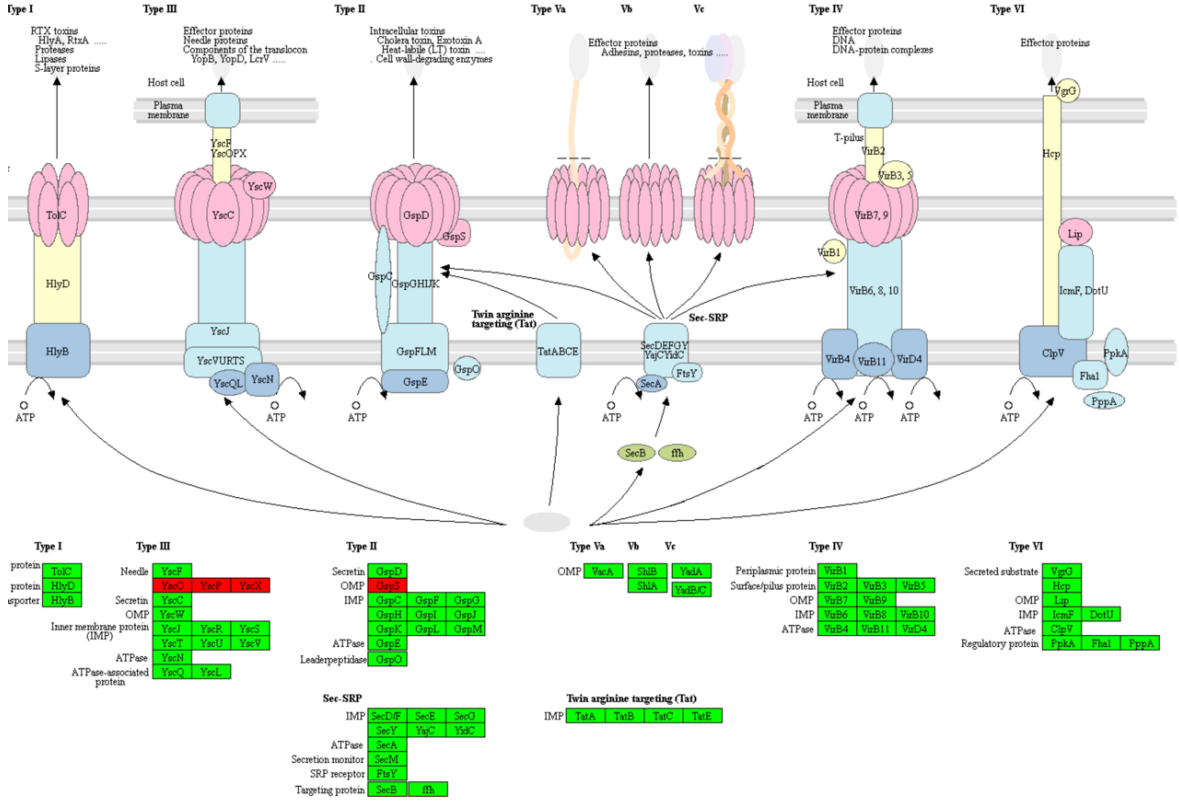


Figure 3-2-13 The KEGG pathway for bacterial secretion systems. The KOs detected in the metagenome sequencing were marked in green, while the missing KOs were marked in red.

3.2.3 Genome reconstruction reveals the link between function and taxonomy

We have reconstructed 25 genomic bins, hereafter referred to as metagenome-assembled genomes (MAGs). The completeness and contamination ratios of these MAGs are listed in Table 3-2-1. According to the standards developed by the Genomic Standards Consortium (GSC) (Bowers et al. 2017), 14 MAGs are high-quality drafts, while the rest 11 MAGs are medium-quality drafts. The reconstructed genomes belong to Paenibacillaceae, Rhizobiaceae, Burkholderiaceae, Comamonadaceae, Enterobacteriaceae, Erwiniaceae, Pseudomonadaceae and Xanthomonadaceae (Figure 3-2-14). Among all the 25 MAGs, 10 reconstructed genomes were assigned to genus level, namely *Agrobacterium*, *Delftia*, *Pantoea*, and *Pseudomonas;* 5 were assigned to species level, namely *Paenibacillus xylanexedens*, *Paraburkholderia fungorum*, *Kosakonia cowanii*, and *Stenotrophomonas rhizophila*.

We further investigated the functional pathways of the MAGs. A few characteristics were found prevalent in the bacterial endophytes, including motility and chemotaxis, plant adhesion and polymer degradation, antioxidant enzymes, osmoprotectant transport, the synthesis of compatible osmolyte trehalose, iron uptake and phytohormone production (Table 3-2-2). Our results show that multiple secretion systems co-exist in the bacterial endophytes. Besides the general Sec and Tat secretion system, type II, type III and type VI secretion systems were most prevalent in the bacterial endophytes.

Table 3-2-1 The completeness, contamination, coverage and phylogeny of the metagenome assembled genomes (MAGs) reconstructed from barley seed-borne root endophytes metagenome

Completion [a]: ratio of observed single-copy marker genes to total single-copy marker genes in chosen marker gene set.
Contamination [b]: ratio of observed single-copy marker genes in ≥2 copies to total single-copy marker genes in chosen marker gene set.
N50 [c]: the minimum contig length needed to cover 50% of the genome. It means half of the genome sequence is in contigs larger than or equal the N50 contig size.

Nr	completeness [a]	contamination [b]	GC	lineage	N50 [c]	size	Cultivar
1	86.48	3.171	0.589	Rhizobiaceae	4958	5610437	Barke
2	97.09	0.887	0.555	Pantoea	25755	4414970	Barke
3	98.01	0.769	0.608	Pseudomonas	45156	5784290	Barke
4	97.57	0.817	0.594	Rhizobiaceae	88174	5720537	Barke
5	99.82	0.228	0.595	Rhizobiaceae	246888	4945027	Barke
6	78.68	1.415	0.672	Stenotrophomonas rhizophila	4523	3527969	Barke
7	99.01	0.355	0.554	Pantoea	32568	4649853	Barke
8	75.88	2.952	0.400	Paenibacillaceae	3130	3763997	Barke
9	94.65	0.066	0.530	Enterobacteriaceae	171824	4260520	Salome
10	99.57	0.464	0.558	Pantoea	34202	4396240	Salome
11	79.85	2.161	0.619	Paraburkholderia fungorum	5455	6032202	Salome
12	96.07	1.447	0.606	Pseudomonas	11811	5458776	Salome
13	73.24	1.648	0.583	Agrobacterium	4064	4050261	Salome
14	97.00	0.560	0.556	Enterobacterales	21500	4618936	Salome
15	98.52	0.327	0.559	Pantoea	27725	4216843	Salome
16	96.82	0.338	0.606	Pseudomonas	21419	5441609	Salome
17	93.63	1.663	0.564	Kosakonia cowanii	12625	4226699	Salome
18	75.98	0.457	0.464	Paenibacillus xylanexedens	4746	4973693	Salome
19	77.93	1.031	0.655	Delftia	4936	4270944	Salome
20	78.36	0.182	0.401	Paenibacillaceae	5439	3661297	Salome
21	100.0	0.086	0.662	Xanthomonadaceae	66158	4544884	Salome
22	97.12	2.890	0.618	Paraburkholderia fungorum	23853	8412798	Salome
23	98.66	0.420	0.556	Pantoea	31726	4484086	Salome
24	81.98	1.121	0.399	Paenibacillaceae	2654	3724337	Salome
25	78.18	8.219	0.540	Enterobacterales	37500	3762724	Salome

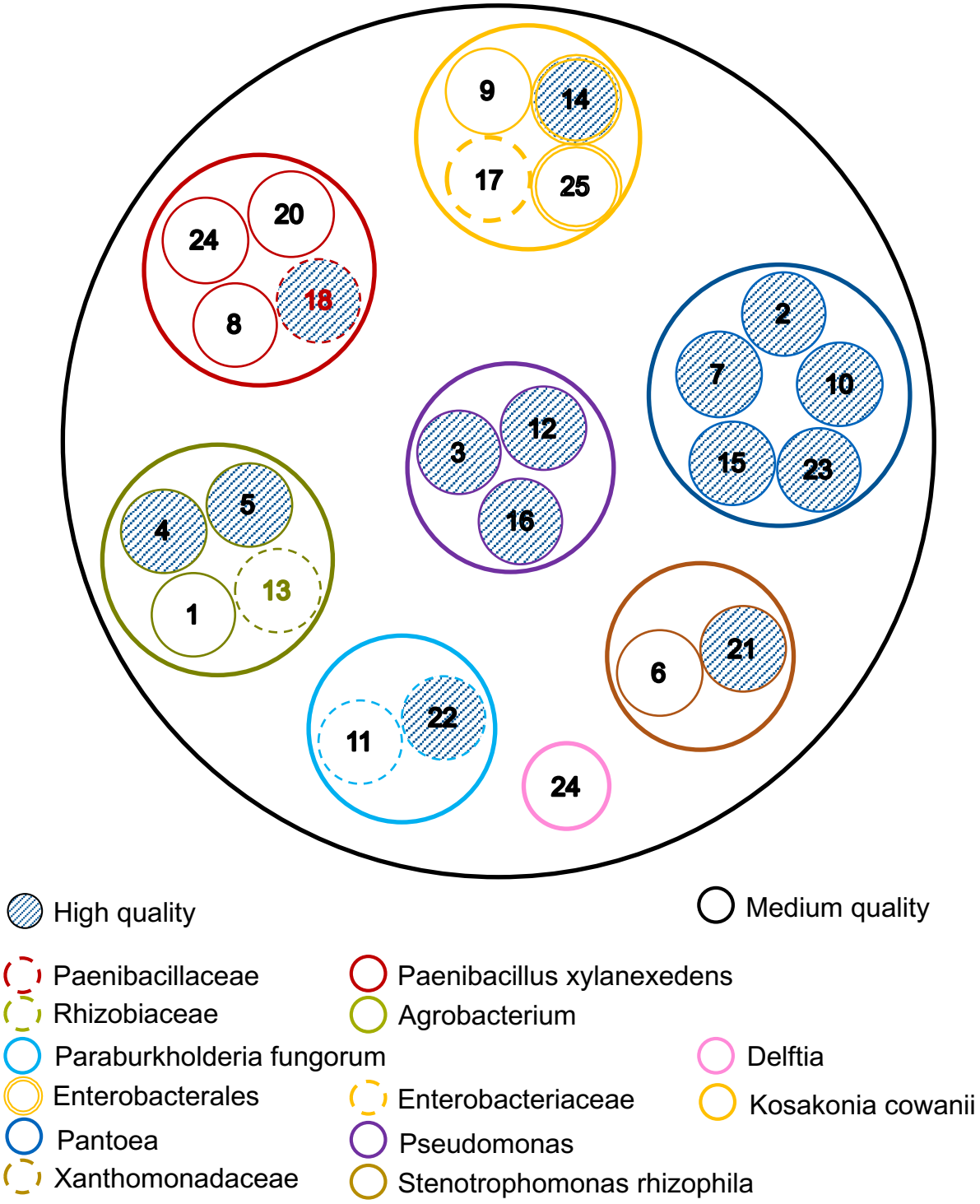


Figure 3-2-14 The illustration of taxonomic lineages of the metagenome assembled genomes (MAGs). Each small circle represents one MAG. The number in the circles corresponds to the serial number of MAGs in Table 3-2-1. The slash filled circles represent high-quality draft and the hollow circles represent medium-quality draft. The colors represent different groups of bacteria. The type of the circle lines indicates the levels of the taxonomic assignment. The double line represents order, the dashed line represents family and the solid line represents genus/species.

Table 3-2-2 The important functions found in the metagenome-assembled-genomes (MAGs). ‘ +’ indicates the presence of relevant functions. ‘ /’ indicates that the rlevant functions were not detected in the investigated MAG. ‘ M’ represents Medium quality draft, while ‘ H’ represents high quality draft. The number of bin corresponds to the serial number of MAGs in Table 3-2-1. P_e: Paenibacillaceae, P.x: Paenibacillus xylanexedens, R: Rhizobiaceae, A: Agrobacterium, P.f: Paraburkholderia fungorum, Del: Delftia, E_es: Enterobacterale, E_ae: Enterobacteriaceae, K.co: Kosakonia cowanii, Pa: Pantoea, Ps: Pseudomonas, X: Xanthomonadaceae, S.r: Stenotrophomonas rhizophila

	Nr of Bin	8	20	24	18	1	4	5	1	11	22	19	14	25	9	17	2	7	10	15	23	3	12	16	21	6	occurrence frequency
	Taxonomy	P_e	P_e	P_e	P.x	R	R	R	A	P.f	P.f	De	E_es	E_es	E_ae	K.co	Pa	Pa	Pa	Pa	Pa	Ps	Ps	Ps	X	S.r	
	Lineage	Paenibacilliaceae				Rhizobiaceae				Burkholderiaceae		Delftia	Enterobacterales		Enterobacteriaceae		Erwiniaceae					Pseudomanadacea			Xanthomonadacea		
	quality	M	M	M	M	M	H	H	M	M	H	M	H	M	M	M	H	H	H	H	H	H	H	H	H	M	
Nutrient aquisition	Amylase	+	+	+	+	+	+	+	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24
	glucosidase	+	+	+	+	+	+	+	+	+	+	/	+	/	+	+	+	+	+	+	+	/	/	+	+	+	21
	Aminopeptidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	25
	Phytase	+	+	+	/	/	/	+	/	/	/	+	+	+	/	/	+	+	+	+	+	/	/	/	+	+	14
	siderphore	+	+	+	+	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	23
	TonB depedent	/	/	/	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	21
	Iron (III)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	25
osmoprectectant	osmoprotectant	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	23
	Trehalose	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24
phytohormone production	ACC deaminase	/	/	/	/	/	/	/	/	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	/	/	14
	IAA	+	+	+	/	/	/	/	/	/	/	/	+	+	+	/	+	+	+	+	+	+	+	+	/	/	14
colonization	methyl accepting	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	25
	Flagellar	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	25
	pili	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24
	adhesion	+	/	+	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	/	22
	Hemaglutinin	+	+	+	+	/	/	/	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	21
antioxidant enzymes	catalase	+	+	+	+	+	+	/	+	+	+	+	+	+	+	+	+	+	/	+	+	+	+	+	+	+	23
	superoxide	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	/	+	+	+	+	24
plant cell wall degrading enzymes	endoglucanase	+	+	+	+	+	+	+	+	+	+	/	+	+	+	+	+	+	+	+	+	/	/	+	/	/	20
	pectinase	/	/	+	/	/	/	/	/	/	/	/	/	/	+	/	+	/	/	/	/	+	+	+	/	/	6
	xylanase	/	+	+	/	+	+	+	+	/	/	+	/	/	/	/	/	/	/	/	/	/	/	/	+	+	9
secretion systems	typeI	/	/	/	/	+	+	+	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	20
	type II	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24
	typeIII	+	+	+	+	+	+	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	24
	typeIV	/	/	/	/	+	+	+	+	+	+	/	/	/	+	/	+	/	/	/	/	/	/	/	+	/	9
	Type V	/	/	/	/	/	/	/	/	+	+	/	/	/	+	/	/	/	+	/	/	/	/	/	/	/	4
	type VI	/	/	/	/	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	20
	sec	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	25
	Tat	/	+	+	/	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	23

3.3 The role of soil microbiome and seed-borne endophytes in barley drought response

3.3.1 Barley plants perform better under drought stress with the help of soil microbiome

To investigate the effects of the microbial community on plant performance, we grew barley in both natural and autoclaved soil. Under regular watering, the water content in the autoclaved soil was lower than that in the natural soil (Figure 3-3-1 (A)). However, no differences were found in the leaves' water content of plants grown in the two soils (Figure 3-3-1 (B)). Moreover, we did not observe significant differences in plant biomass retrieved from the two soils (Figure 3-3-2).

The soil water content from pots submitted to drought stress was significantly lower compared to those from regularly watered pots and did not significantly differ between autoclaved and natural soils (Figure 3-3-1(A)). Plants exhibited visible signs of water deficit, as they showed leaf rolling and the color of leaves turned from green to yellow. Leaves from stressed plants had 50% lower water content compared to control plants (Figure 3-3-1(B)), independent of the status of the soil.

Under drought stress, the total plant biomass of barely grown in natural soil was higher than that in the autoclaved soil ($p<0.05$) (Figure 3-3-2). Those differences mainly reflect higher root biomass for the drought-stressed plants, as significant differences ($p<0.05$) were only found for roots but not for shoots (Figure 3-3-2).

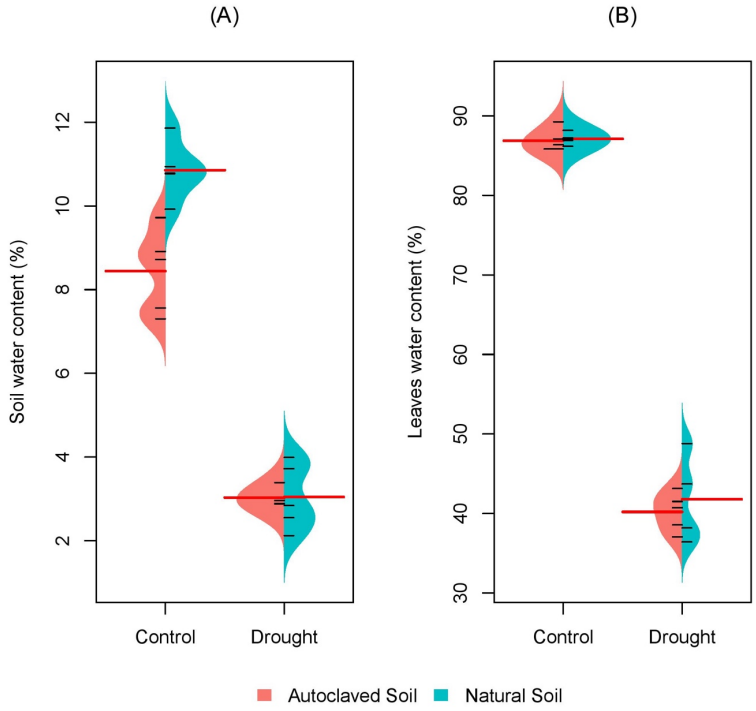


Figure 3-3-1 Water content of (A) soil and (B) leaves in autoclaved and natural soil under control and drought conditions (n=4-5).

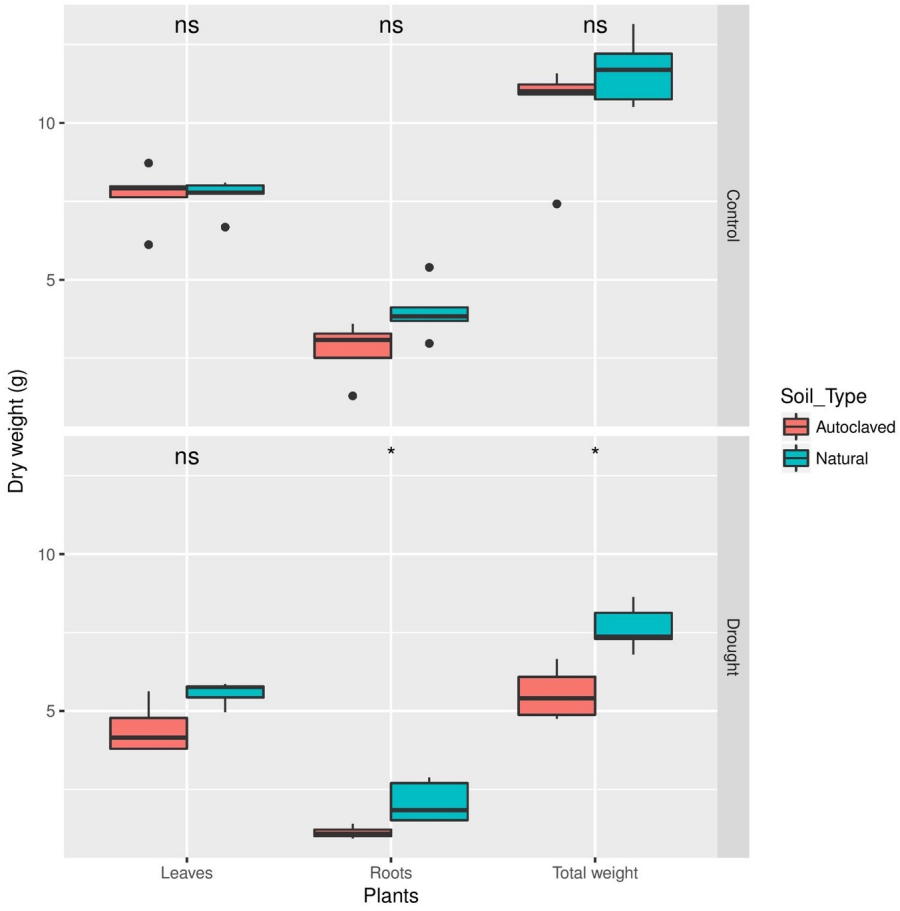


Figure 3-3-2 Plant biomass of barley grown in autoclaved and natural soil under control and drought conditions (n=5) (ns: not significant; *: significant, $p<0.05$)

We also measured the antioxidant enzyme Peroxidase (POX), an indicator of redox status under drought stress. The activity of peroxidase was highly increased in barley leaves under drought stress ($p<0.05$) (Figure 3-3-3). However, no significant differences in POX activity were observed in drought-stressed plants grown in natural and autoclaved soils. After re-watering, plant vigor was partly improved due to drought alleviation. The peroxidase level of barley showed a tendency of decrease in the natural soil, although without statistical significance (Figure 3-3-3). In contrast, this tendency was not observed for the autoclaved soil.

3.3.2 Root endophytic communities under drought stress differ according to soil status

To investigate how root endophytic community was affected by drought, we normalized the OTU table and calculated Shannon and Chao1 index for both bacteria and fungi in each sample. No significant differences of alpha diversity were found for root endophytic bacterial and fungal communities under drought in two soils. (Figure 3-3-4).

We further performed permutational multivariate analysis (PERMANOVA) to evaluate the impact of drought stress and soil status on root endophytes. We did not detect differences in

bacterial and fungal endosphere microbiome caused by the one-week drought. Instead, soil status accounted for the community difference. In accordance, clustering patterns could be observed in the principal coordinate (PCoA) plot of the bacterial community (Figure 3-3-5).

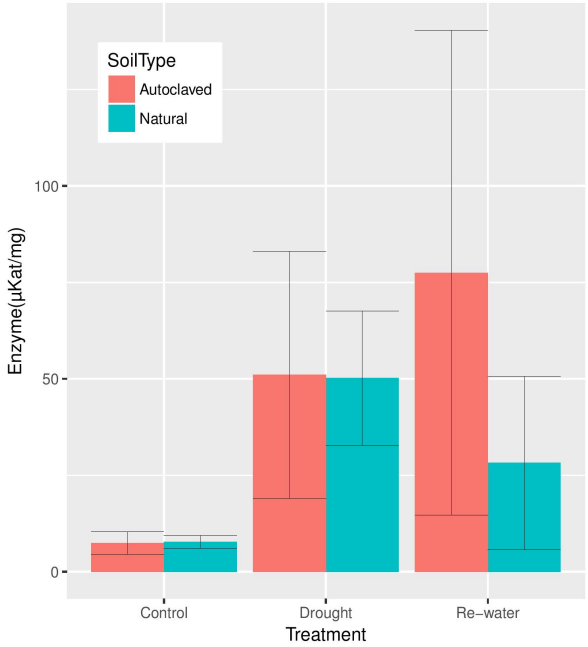


Figure 3-3-3 Peroxidase activity in leaves of barley plants grown in the natural and autoclaved soil under control, drought and drough alleviated (Re-water) conditions (n=5-7)

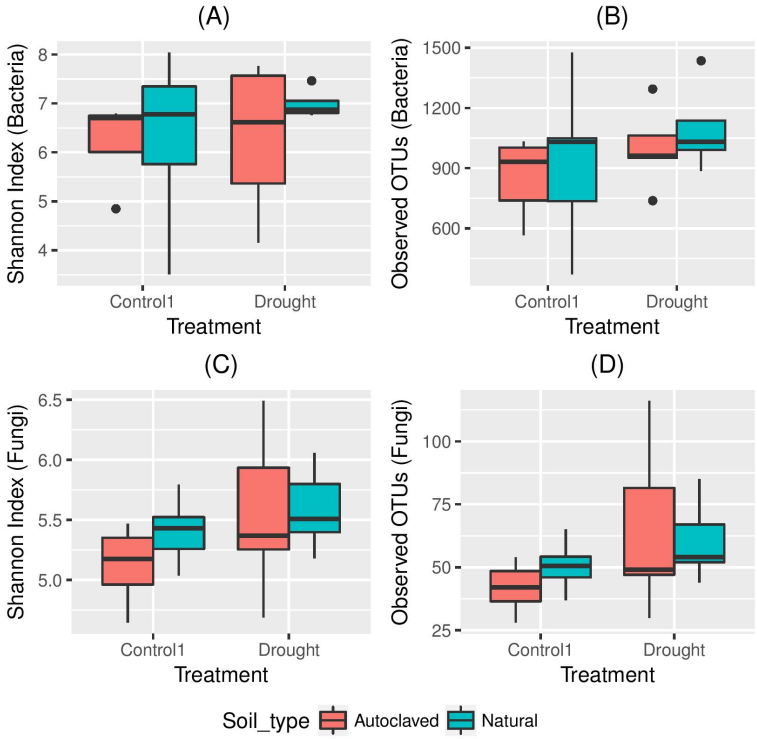


Figure 3-3-4 (A) Shannon index and (B) observed OTUs of bacterial root endophytes; (C) Shannon index and (D) observed OTUs of fungal root endophytes from autoclaved and natural soil under control and drought conditions (n=4-5)

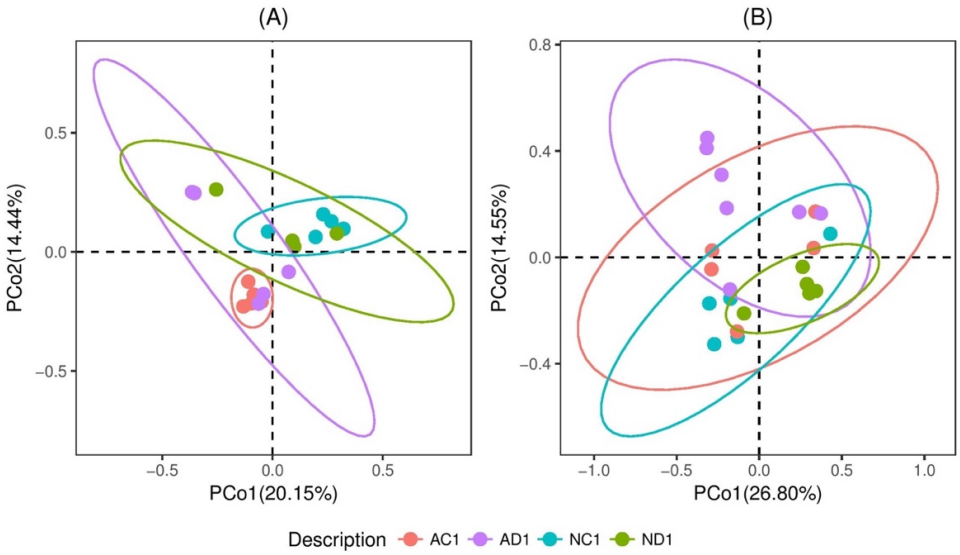


Figure 3-3-5 PCoA plots of root endophytic (A) bacteria using unweighted Unifrac metrics and (B) fungi using Bray-Curtis dissimilarity in autoclaved and natural soil under control and drought conditions (n=4-7). The ellipses represent 95% confidence interval of corresponding samples. (AC1: autoclaved soil under control conditions, AD1: autoclaved soil drought stressed, NC1: natural soil under control conditions,ND1: natural soil drought stressed)

In root endophytic bacteria, the most abundant genera were *Pseudomonas*, *Agrobacterium* and *Stenotrophomonas* (Figure 3-3-6).

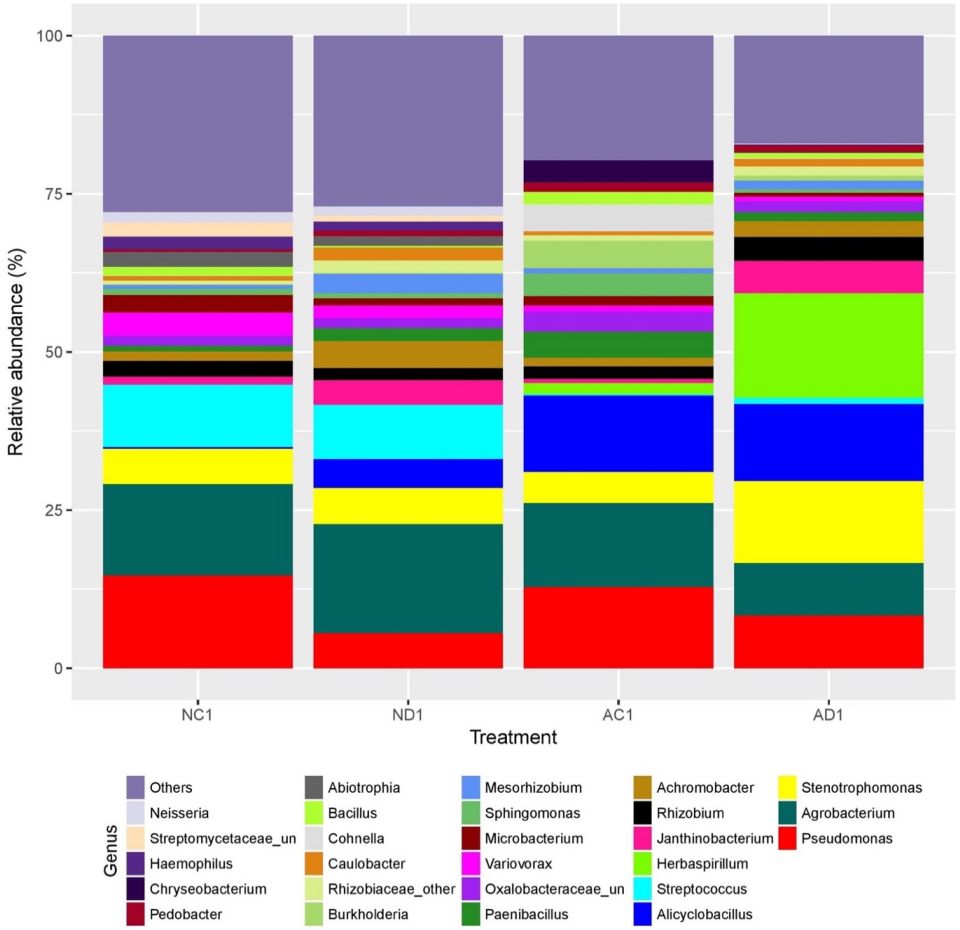
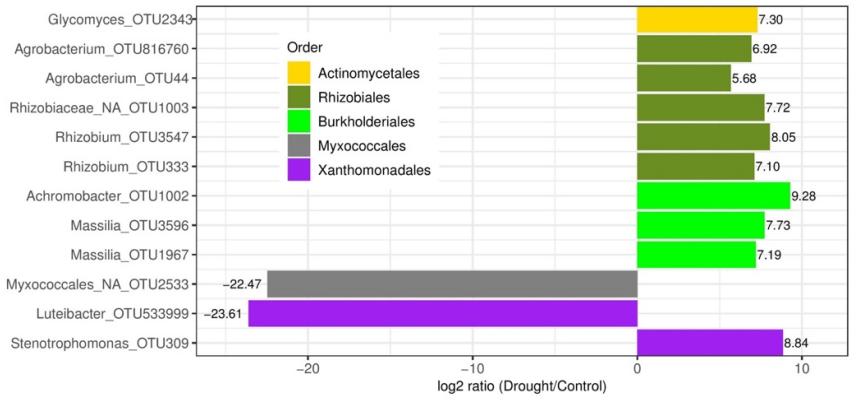


Figure 3-3-6 Major genera of bacterial endophytes in roots grown in the natural and autoclaved soil under regular watering and drought stress (n=4-5) (NC1: natural soil under regular watering, ND1: natural soil drought stressed, AC1: autoclaved soil under regular watering, AD1: autoclaved soil drought stressed)

We normalized the OTU table and searched for OTUs that differ under control and drought conditions using DESeq2. In the natural soil, 12 OTUs differed between the two treatments, among which 10 OTUs were enriched under drought stress. At the level of order, the enriched OTUs spanned from Actinomycetales, Rhizobiales, Burkholderiales to Xanthomonadales. Specifically, the OTUs were assigned to *Glycomyces*, *Agrobacterium*, *Rhizobium*, *Achromobacter*, *Massilia*, and *Stenotrophomonas*. (Figure 3-3-7 (A)).
In the autoclaved soil, 23 OTUs were enriched under drought stress, among which more than half belonged to Rhizobiales and Burkholderiales (Figure 3-3-7 (B)). Notably, the drought-resulted shift in the autoclaved soil differed from that in the natural soil. In the autoclaved soil, the three OTUs belonged to Actinobacteria were depleted in roots under drought, while in the natural soil, the OTU belong to Actinobacteria was enriched in drought-stressed roots. Moreover, four enriched OTUs were found within the order of Enterobacteriales, which were further assigned to *Pantoea* and *Erwinia*. Interestingly, these four OTUs were also detected in barley seeds. Besides, five OTUs depleted under drought stress assigned to *Pseudomonas* were also detected in barley seeds. In contrast, none of the drought-affected OTUs in the natural soil were seed originated.



(A)

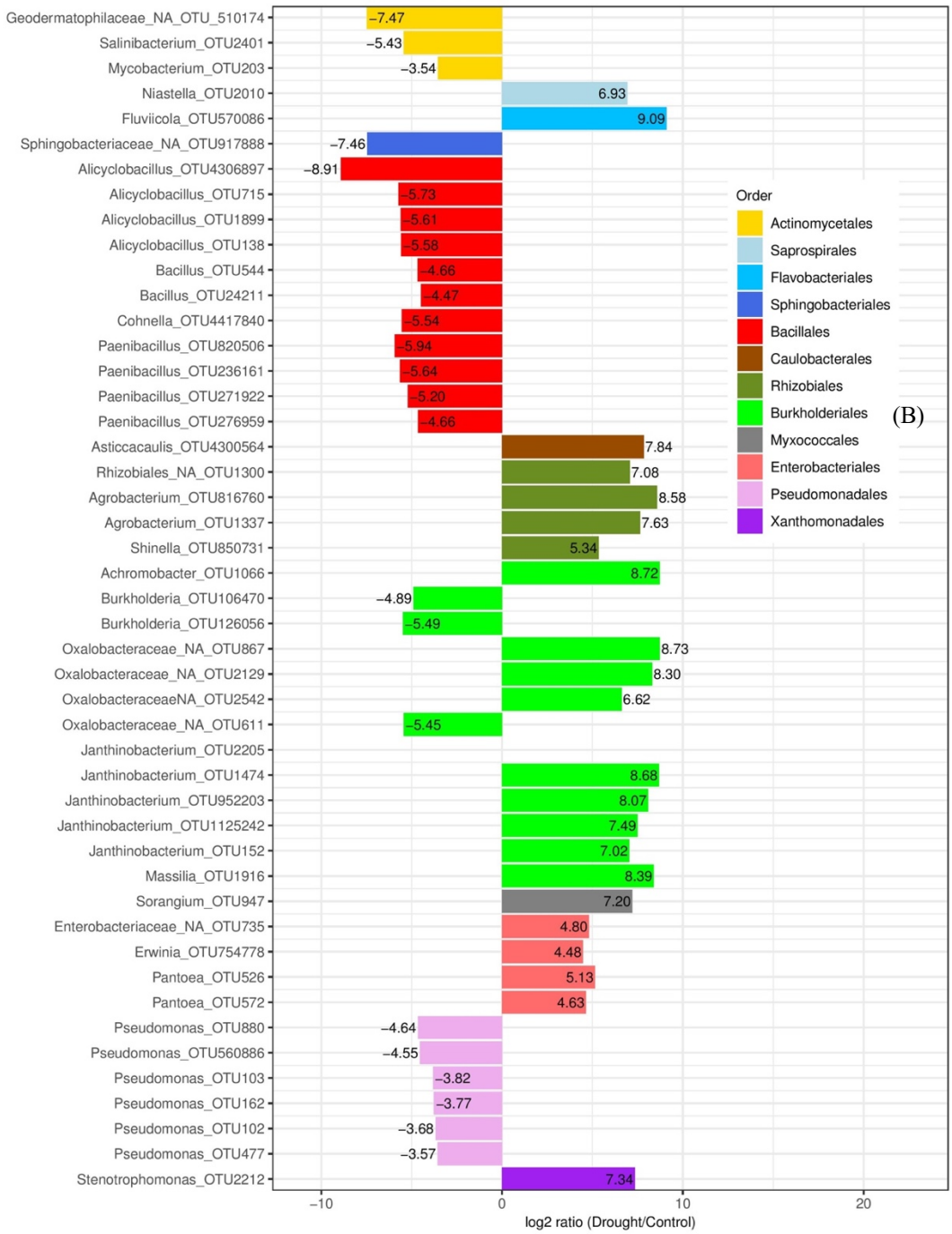


Figure 3-3-7 Bacterial OTUs of barley root endophytes differ under drought and control conditions in (A) natural soil and (B) autoclaved soil. Bars represent differentially abundant OTUs. The number at the end of the bars represent log_2 fold changes comparing drought and control treatment. The colors indicate different orders. (n=4-5)

We further carried out pairwise t-test to verify the pattern at the genus level. In the natural soil, two genera of bacterial root endophytes showed higher abundance in drought-stressed plants, including *Massilia* and one unassigned genus from the family of Rhizobiaceae (Bonferroni corrected $p<0.05$). In the autoclaved soil, only *Massilia* was found enriched in barley roots under drought stress.

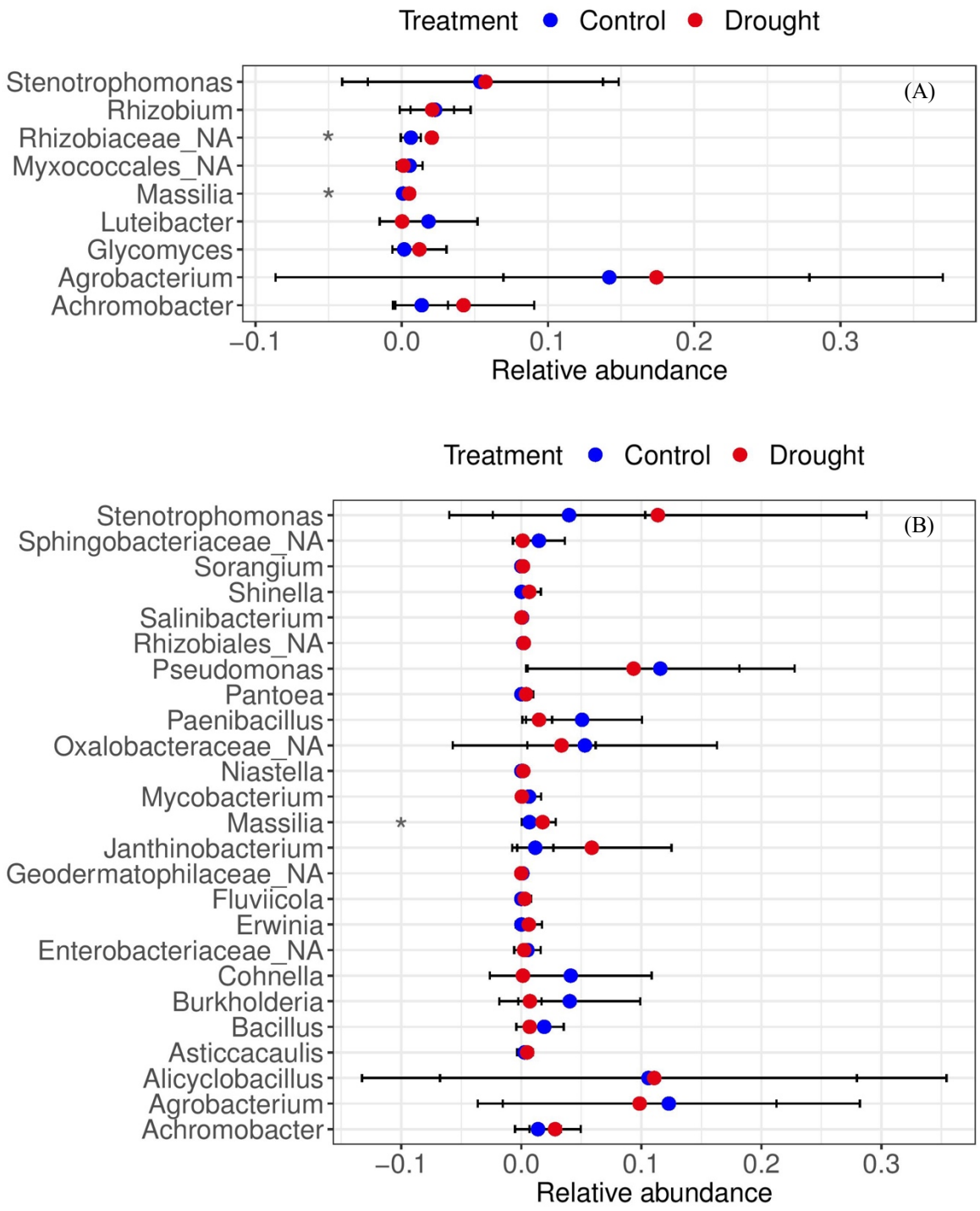


Figure 3-3-8 The relative abundance of genera to which the differentially abundant bacterial OTUs under control and drought treatment were assigned were listed in this figure. Their relative abundance under control and drought conditions were compared in (A) natural and (B) autoclaved soil. Statistical significance is indicated with asterisks (*) (n=4-5).

In the community of fungal root endophytes, most of the assigned fungi belong to Ascomycota while a small fraction was assigned to Basidiomycota. At the genus level, *Fusarium* was the most abundant in all samples regardless of the soil status and watering conditions. *Gibberella* and *Sarocladium* were also in high abundance (Figure 3-3-9).

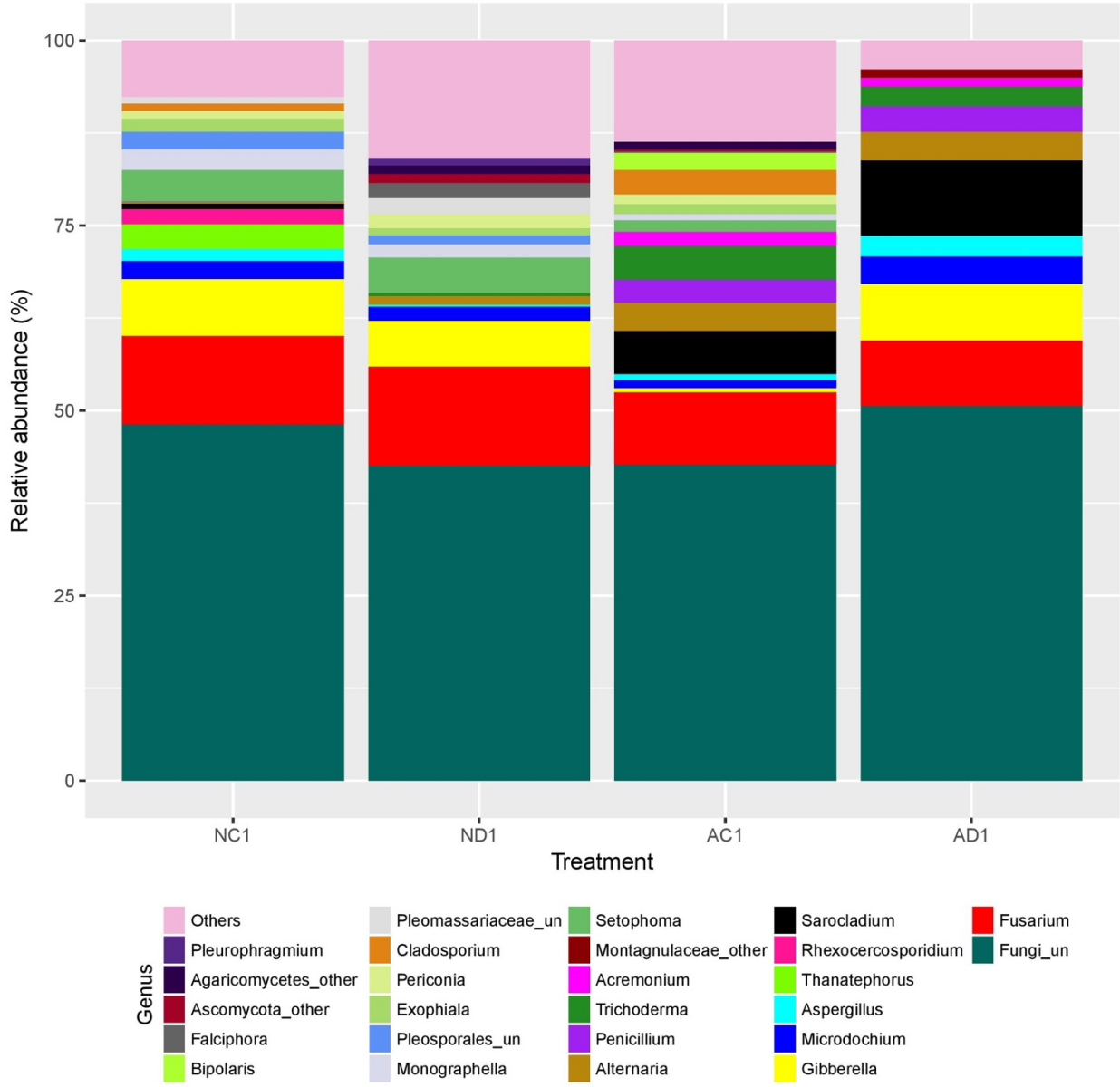


Figure 3-3-9 Major genera of fungal endophytes in roots grown in natural and autoclaved soil under regular watering and drought stress (n=4-5) (NC1: natural soil under regular watering, ND1: natural soil drought stressed, AC1: autoclaved soil under regular watering, AD1: autoclaved soil drought stressed)

We analyzed the fungal OTUs that differ under control and drought conditions using the same method as for bacteria. In the natural soil, only six OTUs were found differ between the two treatments, among which four OTUs were enriched under drought stress. The enriched OTUs were assigned to *Pleurophragmium*, *Falciphora* and two unassigned OTUs in Fungi. (Figure 3-3-10 (A)).

In the autoclaved soil, 23 OTUs differed between control and drought treatment. 19 OTUs were enriched under drought stress, among which around half (nine OTUs) belonged to Nectriacea (Figure 3-3-10(B)).

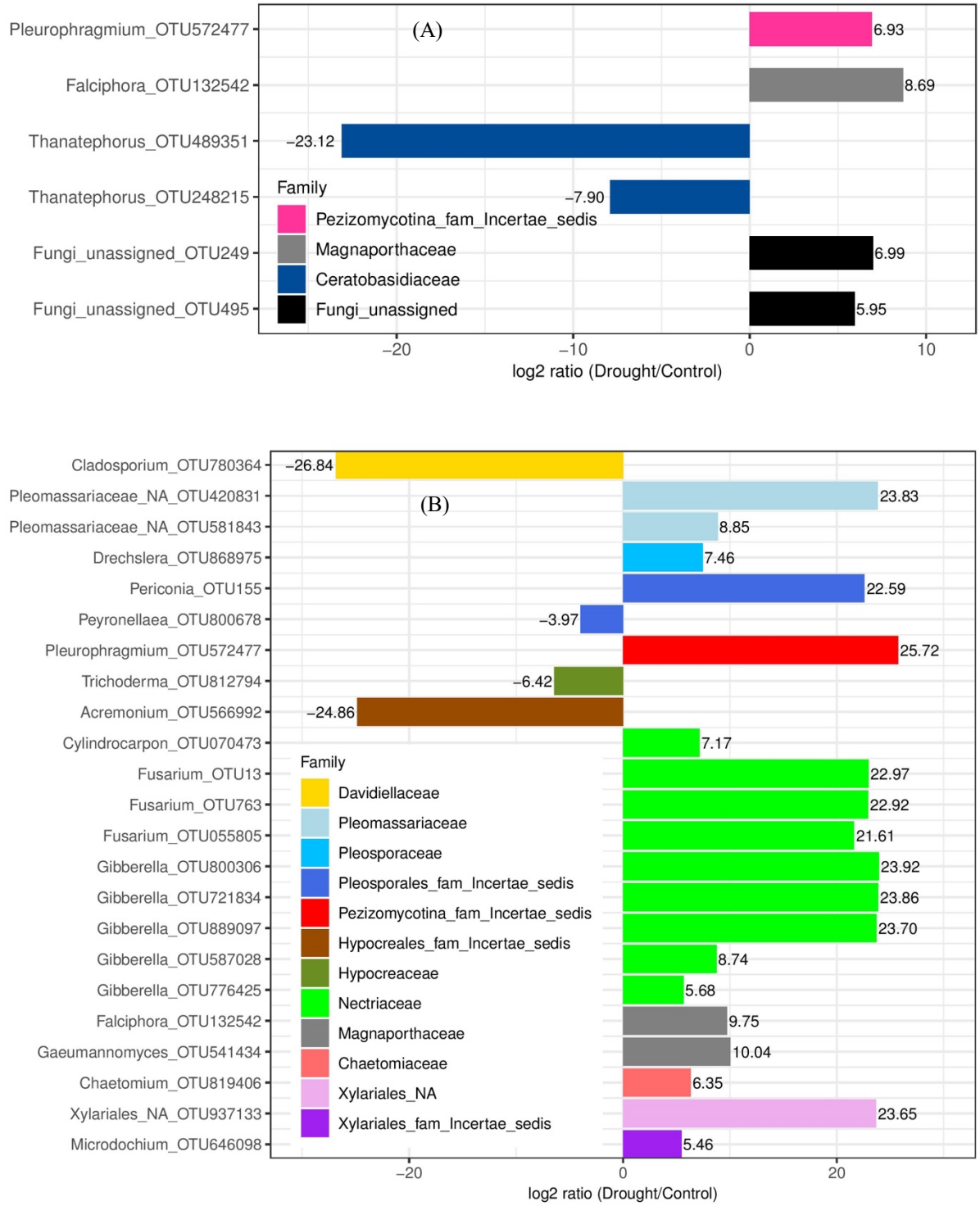


Figure 3-3-10 Fungal OTUs of barley root endophytes differ under drought and control conditions in (A) natural soil and (B) autoclaved soil. Bars represent differentially abundant OTUs. The number at the end of the bars represent $\log_2$ fold changes comparing drought and control treatment. The colors indicate different families. (n=4-5)

We further carried out pairwise t-test to verify the pattern at the genus level. In the natural soil, *Peyronellaea* showed lower abundance in drought-stressed plants (Bonferroni corrected $p<0.05$). In the autoclaved soil, *Gibberella* was found enriched in barley roots under drought stress.

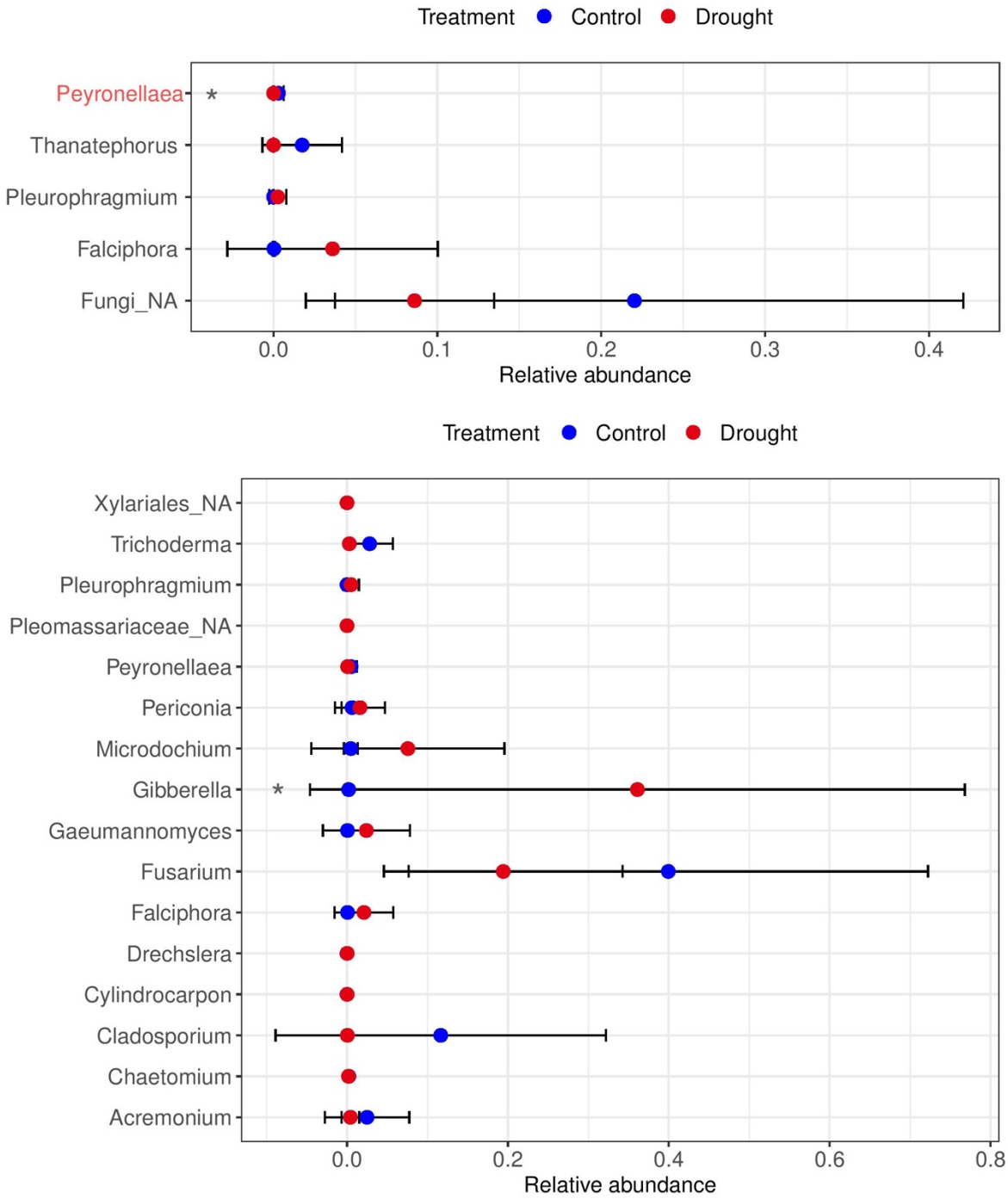


Figure 3-3-11 Pairwise t-test compared the relative abundance of barley fungal root endophytes under control and drought conditions in (A) natural and (B) autoclaved soil. Statistical significance is indicated with asterisks (*) (n=4-5). All the genera to which the differentially abundant bacterial OTUs under control and drought treatment were assigned were listed in this figure. The genus labelled in red represents the genus with statistical significance but not among the genera to which OTUs in Figure 4-11 were assigned.

3.3.3 Root endophytic communities response to re-water differently in normal soil and 'under dysbiosis'

One week after drought stress, the plants were re-watered for two weeks and were thus drought alleviated. No significant differences in alpha diversity were found in root bacterial endophytes from regularly watered and drought alleviated plants (Figure 3-3-12 (A) and (B)). Unlike bacteria, alpha diversity of endophytic fungi communities was significantly higher in drought alleviated plants compared to control plants (Figure 3-3-12 (C) and (D)).
We further compared the alpha diversity of endophytes from the two soil status. Under control conditions, both bacterial and fungal endophytes showed similar diversity in the natural and autoclaved soil. However, after drought and re-water, the alpha diversity of fungal endophytes was higher in roots from the natural soil than the autoclaved soil (Figure 3-3-12 (C) and (D)).

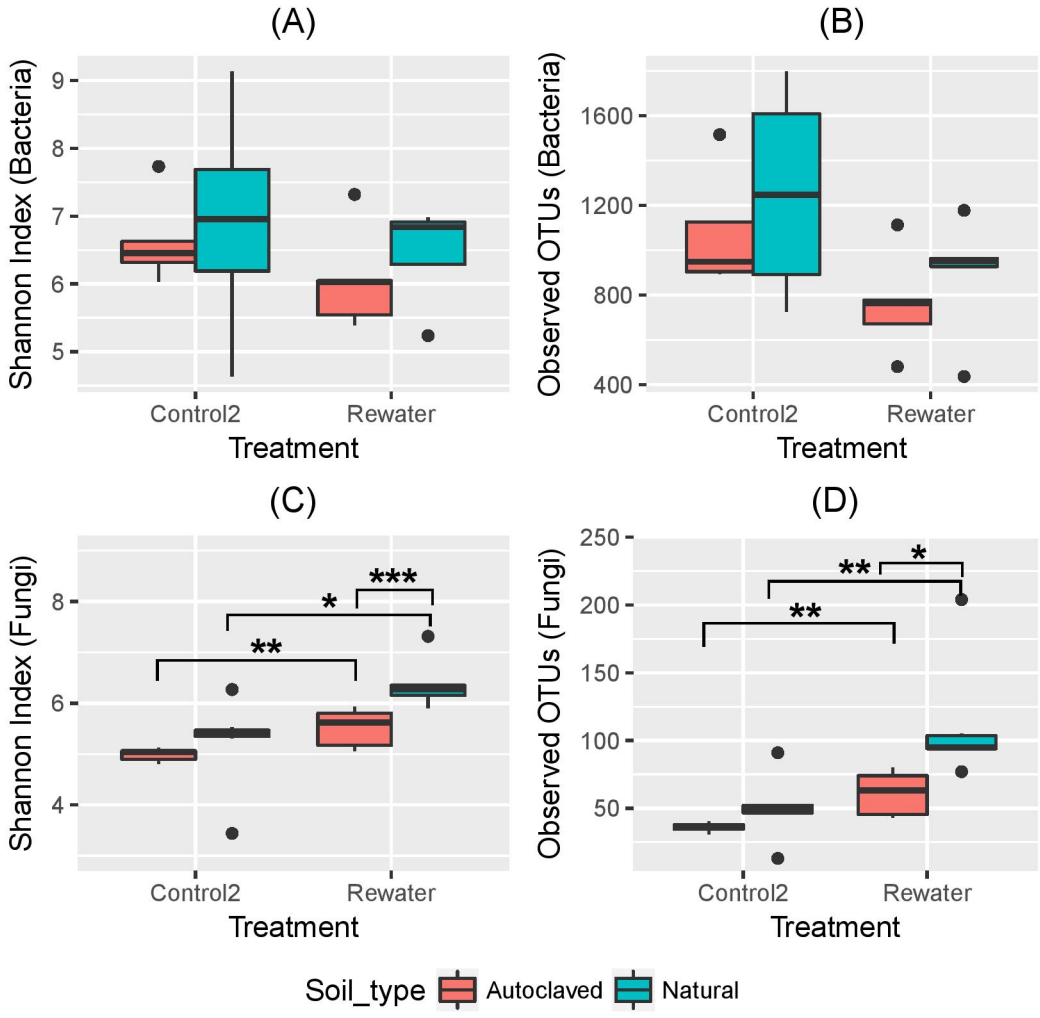


Figure 3-3-12 (A) Shannon index and (B) observed OTUs of bacterial root endophytes; (C) Shannon index and (D) observed OTUs of fungal root endophytes of plants grown in natural and autoclaved soil under control and re-watered conditions (n=5-7) (*:p<0.05, *: p<0.01, ***: p<0.005)

Root endophytic community differed in control and drought-alleviated plants, as revealed by permutational multivariate analysis. Soil status also accounted for the community variation. Clustering patterns could be observed in the PCoA plot (Figure 3-3-13).

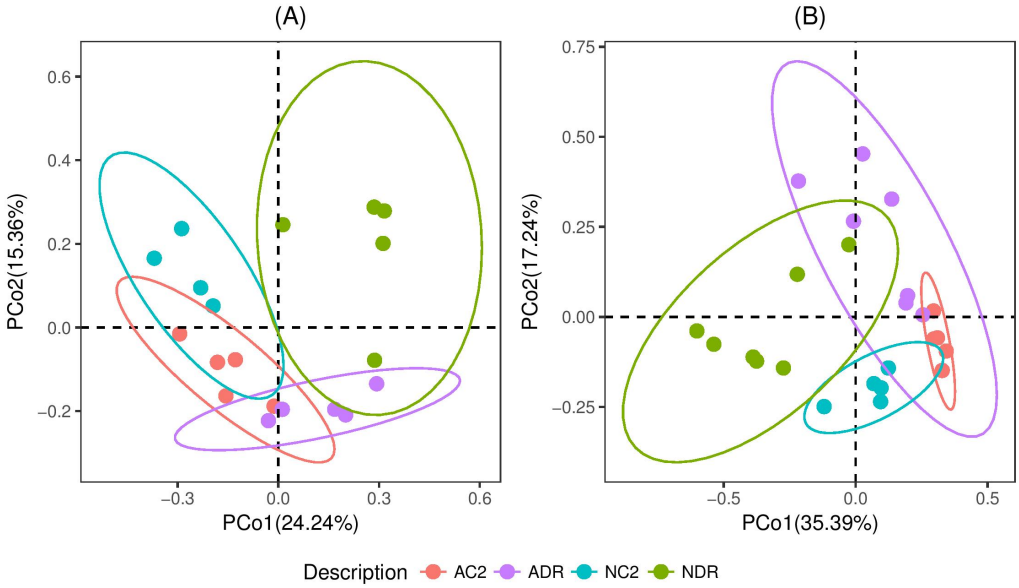


Figure 3-3-13 PCoA plots of root endophytic (A) bacteria using unweighted Unifrac distances and (B) fungi using Bray-Curtis dissimilarity under control and re-watered conditions (n=4-7) (AC2: autoclaved soil under control conditions, ADR: autoclaved soil drought stressed and re-watered, NC2: natural soil under control conditions, NDR: natural soil drought stressed and re-watered)

We further investigated the community differences. Alpha diversity of root endophytes were compared in drought-stressed and drought-alleviated plants. Distinct patterns were observed in the natural and autoclaved soil. Alpha diversity of fungal root endophytes was found higher in the drought-alleviated plants, however, only in the natural soil (Figure 3-3-14).

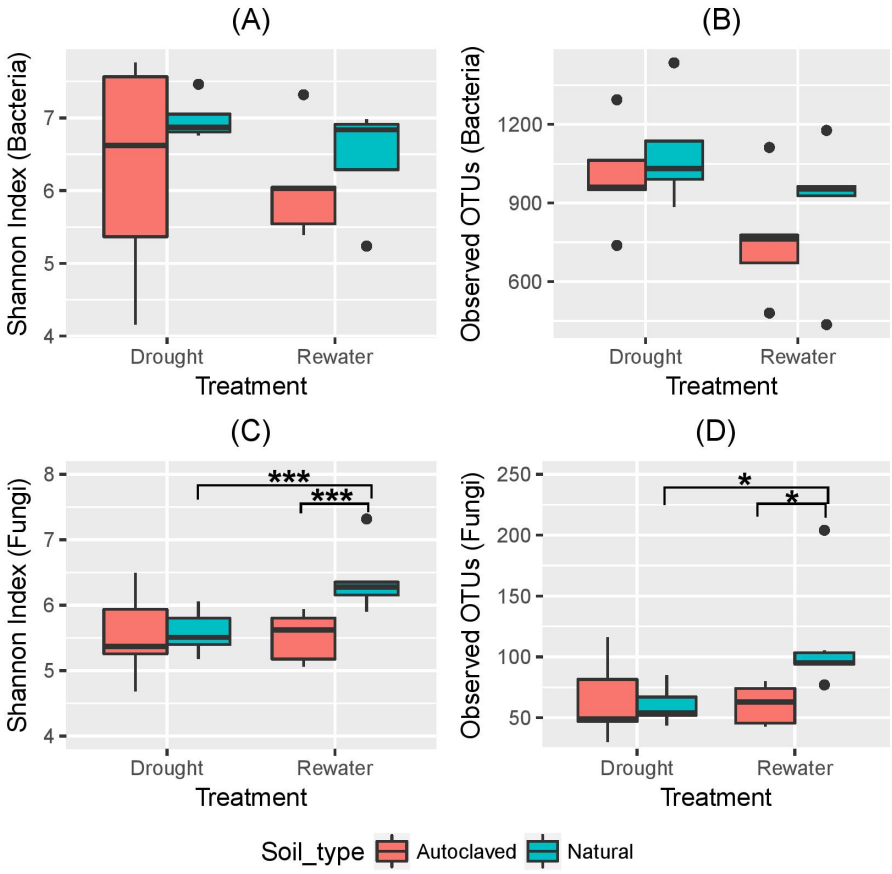


Figure 3-3-14 (A) Shannon index and (B) observed OTUs of bacterial root endophytes;
(C) Shannon index and (D) observed OTUs of fungi root endophytes of plants grown in the natural and autoclaved soil under drought stressed and drought alleviated (re-watered) conditions (n=4-6) (*: $p<0.05$, **:$p<0.01$,***:$p<0.005$)

We further investigated the root endophytic communities in drought-stressed and drought-alleviated plants by calculating the beta diversity. Our results indicate that root endophytic communities respond to re-watering differently in natural and autoclaved soil. In the natural soil, bacterial endophytic community differed significantly between drought-stressed and drought-alleviated plants using both Unifrac distances and Bray-Curtis dissimilarities (Figure 3-3-15). However, in the autoclaved soil, no statistical differences were observed between drought-stressed and drought-alleviated endophytes. Similar results were found in root endophytic fungi. Community differences were observed between drought-alleviated and drought-stressed fungal endophytic community in plants grown in the natural soil, but not in plants grown in the autoclaved soil (Figure 3-3-16).

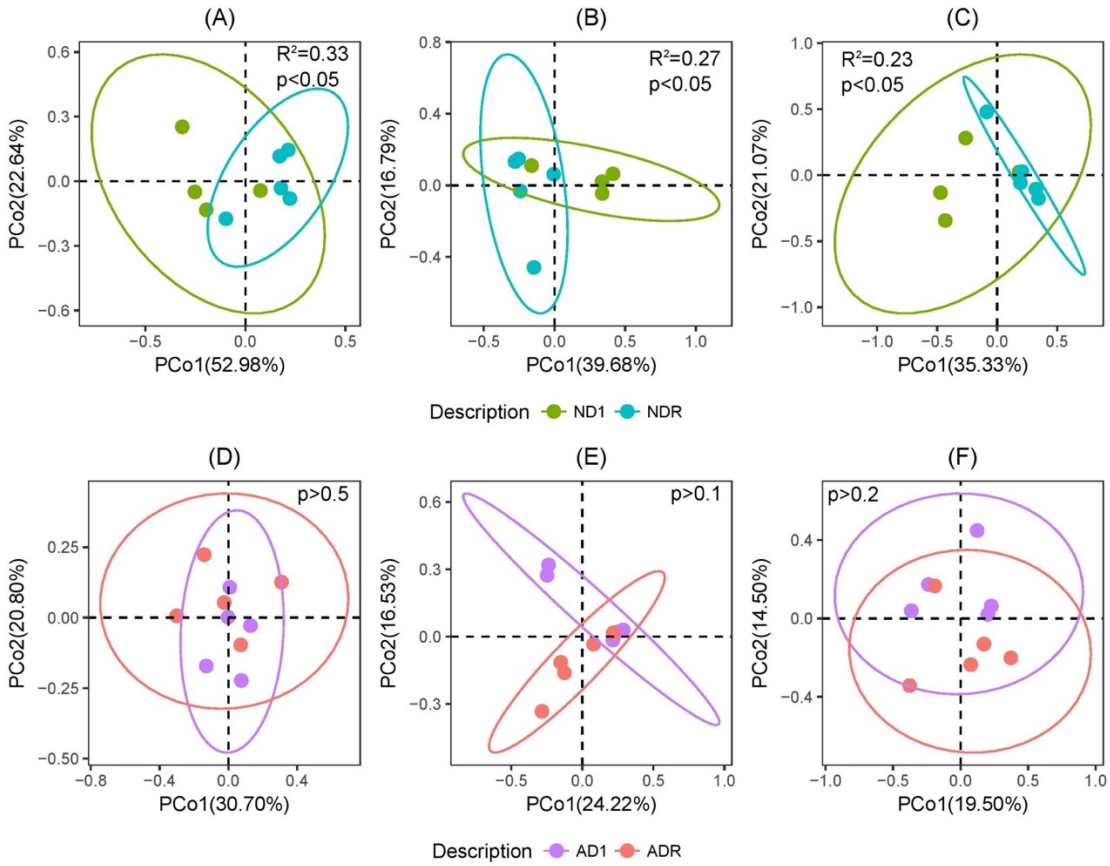


Figure 3-3-15 PCoA plots of bacterial root endophytes in plants grown in the natural soil using (A) weighted Unifrac, (B) unweighted Unifrac and (C) Bray-Curtis dissimilarities; PCoA plots of bacterial root endophytes in plants grown in the autoclaved soil using (D) weighted Unifrac, (E) unweighted Unifrac and (F) Bray-Curtis dissimilarities (n=4-5) (ND1: Natural soil drought stressed, NDR: natural soil drought stressed and re-watered, AD1: autoclaved soil drought stressed, ADR: autoclaved soil drought stressed and re-watered).

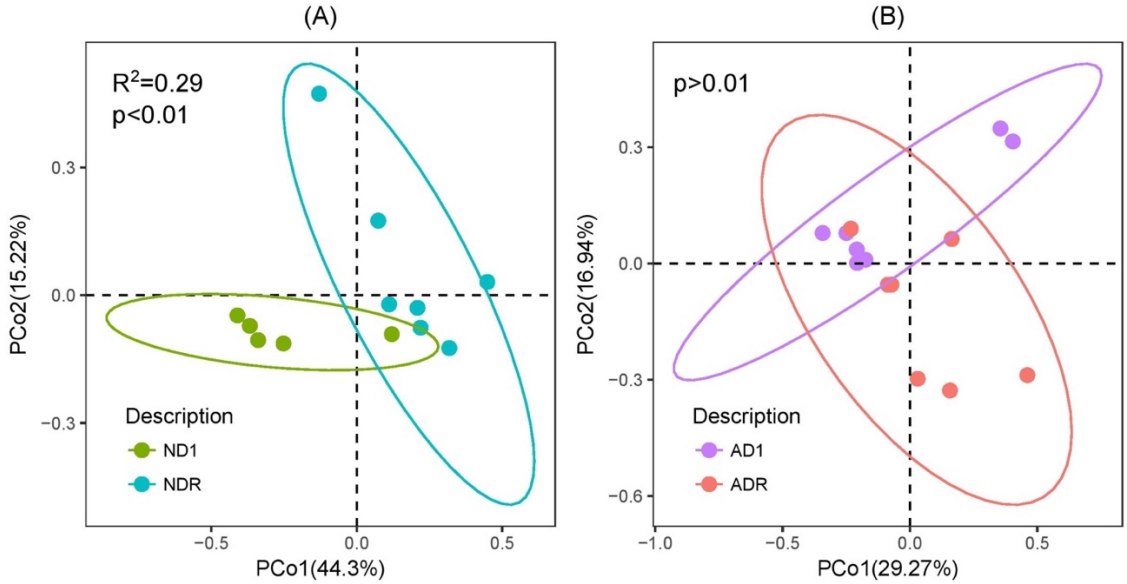


Figure 3-3-16 PCoA plots of fungal root endophytes from (A) natural soil and (B) autoclaved soil using Bray-Curtis dissimilarities (n=5-7) (ND1: Natural soil drought stressed, NDR: natural soil drought stressed and re-watered, AD1: autoclaved soil drought stressed, ADR: autoclaved soil drought stressed and re-watered)

4. Discussion

4.1 The composition and structure of barley seed endophytes

In this study, more than 40 genera of bacteria were detected residing inside barely seeds using amplicon sequencing. The structure of the seed microbiome revealed cultivar-dependent effects. However, around ¾ of the total OTUs were shared by all the barley cultivars in our study. The identification of the large core set of microbial taxa was in consistency with previous studies of the seed endophytes within *Zea* spp., wherein the microbiota was conserved in a variety of wild teosinte ancestors and modern maize across boundaries of evolution, ethnography and ecology (Johnston-Monje and Raizada 2011). The existence of the shared microbiome suggests that the seed endophytes are not casually associated, but rather consist of a selected and conserved community.

Barley seed endophytic community was largely dominated by Enterobacteriacea, followed by Paenibacillaceae and Pseudomonadaceae. A high abundance of Phyllobacteriacea was also observed, however, only in the potentially active groups. At the genus level, *Paenibacillus* and *Pseudomonas* were among the most abundant groups. Surprisingly, *Enterobacter*, the dominant genus of the resident seed endophytes, was negligible in the potentially active groups, indicating that *Enterobacter* are probably not active during seed germination. Instead, we observed the dominance of *Phyllobacterium* in the potentially active seed microbiome. Other genera belonged to Enterobacteriaceae, namely *Erwinia* and *Trabulsiella* also turned out to be in high abundance.

Phyllobacterium has been described as a plant-associated genus and was isolated from the rhizosphere, root, and nodules from different plant species (Mantelin et al. 2006). It was also shown to be vertically transmitted in *Phaseolus vulgaris* (Lopez-Lopez et al. 2010). Although their role in seeds was not clear, *Phyllobacterium* was shown to promote root growth in *Brassica napus* and *Arabidopsis thaliana* (Bertrand et al. 2001; Contesto et al. 2010; Kechid et al. 2013).

Some *Paenibacillus* strains produce cytokinins (Timmusk and Wagner 1999), which are directly involved in seed germination (Kumar et al. 2014) and seed dormancy release (Goggin et al. 2015). Studies have shown that the inoculation of *Arabidopsis thaliana* with a *Paenibacillus polymyxa* strain reduced the germination time (Kefela et al. 2015).

Pseudomonas is widely distributed in rhizosphere and endosphere of plants. They can promote plant growth and drive root development (Devi et al. 2017). Many *Pseudomonas* strains are capable of producing siderophore and thus have a selective advantage over other

bacteria and pathogens, since they overcome competing organisms by depriving them of iron (Chen et al. 2017).
Trabulsiella was also shown to contribute with a great part to the seed microbiome in barley. *Trabulsiella* have been detected in the pollens of *Cupressus* (Fons et al. 2018) and mung sprouts (Naik et al. 2017). However, their roles in plants are not investigated yet.
Many species of *Enterobacter* have plant growth promoting and antifungal abilities (van Dijk and Nelson 1998; Naveed et al. 2014). *Enterobacter cloacea* could suppress plant pathogen *Pythium ultimum* by inactivating the seed exudates that stimulate fungal sporangium germination (Kageyama and Nelson 2003). *Enterobacter* was found to be the keystone species in a model system of the simplified and representative bacterial community of maize roots (Niu et al. 2017).

4.2 The functional potentials of barley seed endophytes

The metagenome sequencing showed a similar profile of the bacterial seed-borne endophytes that vertically transmitted to barley roots as that revealed by amplicon sequencing, except the large dominance of *Pantoea* in the metagenome. Besides, several groups not detected before were revealed in the metagenome sequencing, such as *Koskonia* and *Paraburkholderia*.

In contrast to the cultivar effect in taxonomic composition, no functional differences were detected between the two barley cultivars in our metagenome study. Stable functional structure despite a high taxonomic variability has been observed previously in the microbiota of human guts (Huttenhower et al. 2012), bioreactors (Ofiteru et al. 2010), epiphytes of macroalgae (Burke et al. 2011) and foliage of bromeliad (Louca et al. 2017). This was in line with the paradigm that similar environments should promote similar microbial community function while allowing for taxonomic variation within individual functional groups (Louca et al. 2016; Nelson et al. 2016).

The major functional pathways in the endophytes include chemotaxis, biofilm formation and adhesion, such as methyl-accepting chemotaxis, flagella, pili, and hemagglutinin. Bacterial colonization of root surface is often achieved by flagella and adhesion to plant cells by pili (Mitter et al. 2013). The flagellum filament of invading bacteria is likely one of the first structures to get in contact with plant cells (Bogino et al. 2013). Flagellar proteins or the shorter peptide Fgl22 are common MAMP that trigger plant defense system (Jones and Dangl 2006). However, in another study, flagella were found to be required for efficient endophytic

colonization of rice roots by *Azoarcus* sp. BH72, where flagellin mediated endophytic competence rather than acting as MAMPs (Buschart et al. 2012). Similarly, hemagglutinins have been considered as pathogenicity factors previously, as they were found to be important in both plant and human pathogenic bacteria (Bottcher-Friebertshauser et al. 2014). Their widespread occurrence among bacterial endophytes indicates that hemagglutinin protein may play crucial roles in the invasion of eukaryotic tissues by bacteria in general.

Potential mechanisms of microbial community assembly include adaptation to local environmental conditions also called environmental filtering (Powell et al. 2015). Compared to the endophytes colonizing other plants microhabitats, seed-borne endophyte must withstand high osmotic pressure caused by the accumulation of starch and loss of water during seed maturation (Truyens et al. 2015). In this study, we found that osmo-protectants were widespread in the seed-borne endophytes, suggesting their essentiality for bacterial survival in the seed interior.

The major stored reserves within the seed are mobilized during germination, providing nutrients to support early seedling growth. Starch is the most abundant reserve carbohydrate in cereal seeds, while phytate is the main storage form of phosphorus in seeds. The major storage proteins present in the endosperm are hydrolyzed by proteinases (Ma et al. 2017). Not surprisingly, amylase, aminopeptidase, and phytase were among the most prevalent functional potentials in seed-borne endophytes in our study, which are essential for nutrient mobilization and acquisition.
The siderophore production was found in almost all the MAGs. A high amount of TonB-dependent receptors were found in the MAGs where no siderophore synthesis pathways were detected. It has been suggested that the plant microhabitat is poor in biologically available iron (Morrissey and Guerinot 2009). Thus, the ability of efficient iron acquisition is required for seed-borne endophytes, making them more competitive. Many biocontrol agents utilize this strategy to overcome pathogens by depriving them of iron (Chaiharn et al. 2009).

Our survey of the metagenome data revealed that ACC deaminase and IAA production are widely distributed in seed-borne endophytes which migrated to roots. ACC deaminase could lower the ethylene levels in plants under abiotic stress and is a key mechanism by which bacteria promote plant growth (Li et al. 2015). In the assembled MAGs, the presence of ACC deaminase was only detected in reconstructed genomes assigned to *Paraburkholderia*, Enterobacterales, Enterobacteriaceae, *Pantoea*, and *Pseudomonas*. Unexpectedly, MAGs

which were assigned to Rhizobiaceae lack both ACC deaminase and IAA production functions, indicating that they may employ other means for plant growth stimulation. Indole-3-acetic acid (IAA) is the most common, naturally occurring plant hormone of the auxin class which can promote plant growth (Spaepen and Vanderleyden 2011). However, studies have shown that auxin can crosstalk to plant immune networks (Naseem et al. 2015). It is indicated that bacteria may use IAA to circumvent plant defense reactions. In a recent study of root endophyte fungi *Piriformospora indica*, indole derivative production is not required for growth promotion but for biotrophic colonization of barley roots (Hilbert et al. 2012). Thus, it is possible that the widespread IAA production potential in bacterial endophytes serve as part of their colonization strategy to interact with plants. Gibberellins are involved in the natural process of breaking dormancy and gibberellins in the seed embryo are believed to signal starch hydrolysis (Miransari and Smith 2014). In our metagenome study, gibberellin production pathways were detected, though in low abundance. In the reconstructed genomes, no functions of gibberellin were found. It is possible that the bacteria harboring such functions were in low abundance. An alternative explanation might be that the seed-borne endophytes with such functions did not migrate to roots, even though they were present in the germinating seeds.

Unexpectedly, the plant cell wall degrading enzymes pectinase and xylanase occurred in a low frequency in the MAGs. In contrast, endoglucanase was commonly found. The evolutionary loss of genes involved in degrading the plant cell wall has been well documented for ectomycorrhizal symbioses (Martin et al. 2017). However, the study of nitrogen-fixing endophyte *Azoarcus* sp. strain BH72 demonstrated that endoglucanase is involved in its infection of rice roots. Systemic spreading into the rice shoot and ingress into root epidermis could no longer be detected for its endoglucanase mutant (Reinhold-Hurek et al. 2006). It is possible that endoglucanase is an important determinant for successful endophytic colonization, while pectinase and xylanase are auxiliary. Further experiments are still needed for a thorough understanding of bacterial colonization process.

The secretion of proteins plays an essential role in the interaction of bacteria and plant (Brader et al. 2017). In our study, type VI secretion systems (T6SS) were found to be the most abundant. Similar results were also reported in root endophytes of rice (Sessitsch et al. 2012). T6SS participates in various physiological processes including host infection, bacterial competition, stress response and ion transport (Yang et al. 2018). The T6SS-4 expression is also induced in high osmolarity conditions (Gueguen et al. 2013). It is

speculated that T6SS is essential for the bacterial endophytes to interact with their host and survive in the high-osmotic endosphere.
Besides T6SS, T3SS was also widely occurred in the genomes of seed-borne endophytes transferred to roots. However, the needle part of the T3SS was missing in our whole metagenome sequencing. Similarly, in the metagenome sequencing of rice endophytes, the essential elements of T3SS were missing (Sessitsch et al. 2012). Studies have shown that genes for T3SSs are largely missing or incomplete in genomes of mutualistic endophytes (Reinhold-Hurek and Hurek 2011; Mitter et al. 2013). For example, the genome of the mutualistic strain *Paraburkholderia phytofirmans* PsJN encodes all T3SSs components except the needle-forming protein (Mitter et al. 2013). T3SS is commonly used by symbiotic and pathogenic bacteria to inject effector proteins directly into the host cytoplasm and, thereby, to modulate the host response (Wagner et al. 2018). Due to the absence/incompleteness of T3SS in many endophytes, nonpathogenic endophytes were proposed to be disarmed pathogens (Reinhold-Hurek and Hurek 2011). Recent findings suggest that a loss in functional T3SSs enables the evolution of an endophytic lifestyle.

In summary, our study revealed that the seed-borne endophytes which transferred to roots possess functional potentials including plant growth promotion, endophytic competence, and prerequisites to survive the unique seed environment. Our results cast light on the long-standing question of whether seed endophytes are selected by the host for the benefits of next generation or bacterial endophytes merely use seeds as a vector for dissemination. The metagenome sequencing in our study suggests that these options are not mutually exclusive.

4.3 barley seed endophytes influence root microbiome

In this study, we demonstrated that seeds are important sources of root microbiome. This was evidenced by experiments with barley grown in the axenic systems. Under sterile conditions, a great variety of endophytes were found within barley roots. The major groups were similar to the profile of seed endophytes. Taxonomically, bacteria within the seedling roots were all retrieved from seeds, indicating that bacteria colonizing the seed interior could infect the subsequent generation and become endophytic species in barley plant.
Significant cultivar differences were observed in root endophytes of plants grown in the axenic systems. Compared to seeds, we noted a shift in the taxonomical composition. *Phyllobacterium*, highly abundant in the seeds, were negligible in roots in the axenic systems. On the other hand, bacteria belonging to the genera *Pseudomonas* and *Trabulsiella* were

found largely enriched in root tissue. Two major OTUs, OTU 791973 (*Pseudomonas*) and OTU 725048 (*Trabulsiella*), were found in all root and seeds samples. Many strains of these two families were reported to promote plant growth and were frequently described to be found in roots as well (Bulgarelli et al. 2013; Cope-Selby et al. 2017).

In contrast to the strong cultivar effect in the seed endophytes, cultivar dependent effects were less pronounced in roots grown in soil and were only significant when calculating the distance between samples using weighted Unifrac metrics. Our results indicate that the divergence of root microbiota across cultivars is only quantitative. The variation between the cultivars was manifested in the abundance of many OTUs from diverse taxa, rather than by the presence/absence of single OTUs in the given genotypes. These findings are in accordance with a recent study comparing the resident root microbiota of wild and domesticated barley, where a small but significant host genotype effect on the basis of abundance was reported (Bulgarelli et al. 2015). We suppose that the genetic variation across our genotypes is smaller than that in the above study which compared wild and domesticated barley. Therefore, less variation of the associated microbiome is expected.

Compared to the cultivar, our study indicated that developmental stages contribute more to the variance in the microbial community associated with roots. An increase of bacterial diversity was also observed as barley aged, indicating increasing active bacteria during barley development. In plants grown in arable soil, seed-borne endophytes still constitute an important part of the bacterial communities in root microbiome, as more than half of the OTUs in seeds were recovered in roots at both seedling and booting stages. However, the abundance of the shared OTUs was largely decreased as plants developed. Particularly, we noted that the dominant groups in seeds, *Enterobacter*, *Erwinia*, *Paenibacillus*, and *Phyllobacterium* were less abundant in root endophytes. Our results indicate that bacteria originated from barley seeds became a minor population in the root endophytes of mature plants, which is likely caused by various bacterial species invading from the rhizosphere to the root. It is also possible that seed-borne endophytes spread into shoots, where there is less competition than in the roots, as suggested by previous studies of rice and wheat (Kaga et al. 2009; Hardoim et al. 2012; Mitter et al. 2017). Interestingly, OTUs found in the roots of all plants grown in arable soil were in low abundance and differed from those detected in the axenic systems. The barley plants grown in the same soil were colonized by bacteria belonging to the same taxa, but not the same OTUs. This might be a reflection of the great diversity and functional redundancy found in soils.

We observed an enrichment of Actinomycetales, Rhizobiales and Burkholderiales. Actinobacteria are known to produce several secondary metabolites that may hamper the

growth of other bacteria, including plant pathogens. They were also shown to be enriched in the endophytic compartments of *Arabidopsis thaliana* (Bulgarelli et al. 2012). Moreover, we found that Actinobacteria was in much lower abundance in roots in the axenic systems, indicating that they were enriched from the soil. Rizobiales and Burkholderiales contain many beneficial species with plant growth promoting effects. Notably, the core OTUs shared by all the investigated cultivars also belonged to the order of Actinomycetales, Rhizobiales and Burkholderiales. It is postulated that their persistent occurrence and high abundance might be related to their plant growth promoting and biocontrol ability frequently reported in the literature.

4.4 drought response of barley endophytes

In this study, autoclaved soil was used to represent soil in microbiota dysbiosis. We used natural and autoclaved soil to study the role of the whole soil microbiome on barley drought response, as root microbiome was shown to be largely recruited from the surrounding soil. Our work represents an important step in understanding how the soil microbiome interacts with plants and influences plant performance and stress tolerance.
Although no clear differences were detected under well-irrigated conditions, higher plant biomass and particularly higher dry weight of roots were observed in barley from natural soil under drought stress. The weight and volume of plant roots were reported to be associated with drought resistance of crops (Henry et al. 2011). In two other studies inoculating beneficial strains, plant growth promotion was observed only under water deficit conditions (Rolli et al. 2015; Xu et al. 2018). Our results together with these findings corroborate to the intriguing hypothesis that plant-microbe interactions confer stress tolerance in plants, while less interaction might be required for plant's performance under ideal growth conditions, which normally do not exist. Root microbiome as a plant's extended microbiota can respond to environmental perturbations (Rosenberg and Zilber-Rosenberg 2018), and thus cause adaptive phenotypic plasticity of plants (Goh et al. 2013).

Under drought stress, OTUs belonging to Rhizobiales and Burkholderiales were enriched in root endophytes, namely *Agrobacterium*, *Rhizobium*, *Achromobacter*, and *Massilia*. OTUs assigned to *Stenotrophomonas* were also found enriched. Such enrichment was observed in barley plants grown in both natural and autoclaved soil, indicating a conserved drought response. Notably, the relative abundance of the genus *Massilia* was increased following exposure to drought. Many members of the drought enriched taxa were shown with putative

plant growth-promoting properties and/or drought tolerance. *Massilia* is characterized as aerobic, gram-negative flagellated rod-shaped non-spore forming bacteria (Ofek et al. 2012). Members of *Massilia* were shown to possess the capability to withstand extreme dry conditions, since many have been isolated from arid environments, including desert soils (Ren et al. 2018). *Rhizobium* and *Agrobacterium* are known with the potential for plant growth promotion. The ACC-deaminase-producing strain *Achromobacter piechaudii* ARV8 significantly increased the fresh and dry weights of both tomato and pepper seedlings and reduced the ethylene production under drought stress (Mayak et al. 2004). One strain of *Stenotrophomonas* (*S. rhizophila*) synthesizes and accumulates the compatible solutes glucosylglycerol and trehalose under salt stress conditions (Hagemann et al. 2008). Plant growth promotion and enhanced stress tolerance were observed with the *Stenotrophomonas* strain (Rolli et al. 2015). We speculate that the common taxa in droughted roots might be selected by plants based on the specific plant growth-promoting traits to help plants better survive the stress. However, further experiments are needed to test if this selection is ultimately beneficial for the host.

Alternatively, drought-induced plant responses, including root traits, exudation patterns, or changes in niche opportunities on and inside the root surface, could be responsible for shifts of the endophytic microbiome. One putative mechanism for this selection could include shifts in cell wall biochemistry, as plants are known to modulate cell wall components in response to drought (Le Gall et al. 2015).

Despite the common taxa, drought-induced changes of root endophytes showed different patterns in natural and autoclaved soil. Particularly, we noted the enrichment of the OTU belonging to Actinobacteria in the natural soil under drought stress, which was in accordance with a few studies published recently (Naylor et al. 2017; Santos-Medellin et al. 2017). Actinobacteria are known with the ability to survive in arid regions (Mohammadipanah and Wink 2016). The features of spore production and thick peptidoglycan cell walls may render Actinobacteria drought-resistant (Xu et al. 2018). However, following drought treatment, OTUs belonging to Actinobacteria were depleted in root endophytes in autoclaved soil. As root community differed in the two soils under control conditions, we speculate that the contrasting shifts under drought stress could be due to the community difference before drought treatment.

Noteworthy, our assessment revealed that four drought-enriched OTUs in root endophytes were found in barley seeds, indicating their seed origin. Nevertheless, the phenomenon was only observed in plants from the autoclaved soil. We have demonstrated in our study that root

microbiome originates from both seeds and the surrounding soil. Thus, it is not surprising that seed-borne endophytes may play more important roles in the root of plants grown in the autoclaved soil.

The drought-enriched seed-associated OTUs were assigned to *Pantoea* and *Erwinia*, which were dominating in barley roots grown in sterile substrates. Besides, many drought-related genera were also found in barley seeds, although not the same OTUs. Both *Massilia* and *Stenotrophomonas* were among the core members of barley seed-borne endophyte. Other genera like *Rhizobium*, *Agrobacterium*, *Achromobacter*, and *Janthinobacterium* were also present in seeds. The drought-related lineages are phylogenetically close to taxa found in seeds.

Our results indicate the possible origin of the seed-borne endophytes in the droughted root microbiome. Both seed maturation and droughted roots were subjected to water loss. Thus, similar selective pressure may exert upon endophytes within seeds and droughted roots, which favors bacteria that are tolerant to high osmotic pressure. Indeed, the characteristics of desiccation-tolerance and plant growth promotion have been observed in the identified groups shared by seed-borne and drought-related endophytes.

In the case of fungi, only four OTUs were enriched under drought stress in fungal root community in plants grown in the natural soil, among which two OTUs were unassigned. Although we used an optimized combination of ITS primers, a large fraction of reads remained unclassified. The poor taxonomic classification of fungal OTUs impeded a better/complete elucidation of drought effect on the fungal community. However, in another study where bacterial endophytes revealed extensive taxonomic restructuring after three weeks of drought, no significant changes were observed in the fungal community as well (Santos-Medellin et al. 2017). Studies have shown that fungal networks are more stable than bacterial communities under drought stress (de Vries et al. 2018). Fungi are known to be significantly drought resistant. Many studies revealed that fungal endophytes could mediate plant drought stress tolerance through photosynthesis stimulation, energy releasing and enhanced antioxidative capacity (Ghabooli et al. 2013; Giauque and Hawkes 2013). A meta-analysis of published articles showed that the influence of fungal endophytes on plant performance is dependent on plant water status. While under non-stressed conditions, the overall effect of fungi on plants was mostly neutral, under water-stressed conditions, fungal endophyte showed significantly positive or neutral effects on plants (Dastogeer 2018).

The drought-enriched OTUs in the natural soil, which were assigned to *Pleurophragmium* and *Falciphora* respectively, were also enriched in roots grown in the autoclaved soil.

Besides, many enriched root endophytes under drought stress of barley plants grown in autoclaved soil were found to be potential pathogens, for instance, *Gibberella*. The enrichment of pathogens might result from fewer competitors in the autoclaved soil. Yet it is also possible that the enriched fungi are seed-borne endophytes, which are often similar to pathogens (Geisen et al. 2017).

5. Conclusions

In this thesis, we characterized bacterial communities associated with seeds and roots from five commercially available barley cultivars. The cultivar was found as a significant driving factor in shaping the seed associated microbiome. Yet we still identified a large core set of microbial taxa in seed endophytes, including *Enterobacter*, *Paenibacillus*, *Pseudomonas*, *Massilia*, *Erwinia*, *Pantoea*, *Trabulsiella*, and *Phyllobacterium*.

In this study, we clearly demonstrated that seed endophytes are an important inoculum for bacterial communities in the roots. Under sterile conditions, the major groups were similar to the profile of seed endophytes, indicating the vertical transmission of endophytes from seeds to barley roots. Although cultivar effect was observed in the taxonomic composition of root endophytes in the sterile system, no functional differences were detected between the barley cultivars by metagenome sequencing.

Our study revealed that the seed endophytes vertically transmitted to roots possess functional potentials including plant growth promotion, endophytic competence, and prerequisites to survive the unique seed environment. The most prevalent functions include chemotaxis, biofilm formation and adhesion, such as methyl-accepting chemotaxis, flagella, pili, and hemagglutinin. Other dominant functional pathways were found to be osmoprotectants and antioxidant enzymes pivotal to survive the high osmotic pressure as well as amylase, aminopeptidase, and phytase, which are critical for nutrient mobilization during seed germination. ACC deaminase and IAA production were widely distributed in seed-borne endophytes. The most abundant secretion system is the Type VI secretion system (T6SS). T3SS was also widely occurred. However, the needle part of the T3SS was missing.

When plants were grown in soil, the developmental stage was found to have a more pronounced impact on the community composition, whereas the cultivar effect was only quantitative. Seed endophytes still constitute an important part of the bacterial communities in root microbiome. However, the abundance of the seed OTUs was largely decreased as plants developed. Instead, we observed an enrichment of Actinomycetales, Rhizobiales and Burkholderiales in barley root endophytes. Two OTUs assigned to *Propionibacterium* and *Mesorhizobium* were found in all seeds and roots tissues independent of the plant development stage.

Under well-irrigated conditions, barley plants showed similar performance in natural and autoclaved soil. However, under drought stress, higher plant biomass and particularly higher root dry weight were detected in barley from natural soil. Under drought stress, OTUs belonging to Rhizobiales and Burkholderiales were enriched in root endophytes regardless of the soil status, which were assigned to *Agrobacterium*, *Rhizobium*, *Achromobacter*, and *Massilia*. The OTUs belonging to Actinobacteria were enriched in root endophytes in the natural soil under drought stress, but were depleted in root endophytes in the autoclaved soil. Noteworthy, four drought-enriched OTUs found in root endophytes originated from seeds. The drought-enriched seed-associated OTUs were assigned to *Pantoea* and *Erwinia*, which were dominant in barley roots grown in sterile substrates.

In summary, our results showed not only the enormous microbial and metabolic potential of seed-borne endophytes but also their importance on root microbiota assembly and plant fitness. Our study uncovered the response of root endophytes to drought stress and implicated the potential significance of seed-borne endophytes on plant drought tolerance.

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Supplementary Tables

Table S1 Summary of the relative abundance and taxonomy assignment of the core OTUs in the resident groups of barley seed endophytes

Table S2 Summary of the relative abundance and taxonomy assignment of the core OTUs in the potentially active groups of barley seed endophytes

Table S3 Summary of the relative abundance and taxonomy assignment of the core OTUs in resident root endophytes in axenic systems

Table S4 The average abundance and taxonomy assignment of the core OTUs shared by barley resident seed and root endophytes

Table S5 The relative abundance and taxonomy assignment of the core OTUs in the potentially active barley root endophytes

Table S6 The average abundance and taxonomy assignment of the core OTUs shared by the potentially active seed and root endophytes

Table S7 The relative abundance and taxonomy assignment of the core OTUs in the potentially active root endophytes at seedling stage

Table S8 Summary of the relative abundance and taxonomy assignment of the core OTUs in the potentially active root endophytes at booting stage

Table S9 The relative abundance and taxonomy assignment of the core OTUs shared across all cultivars and both growth stages in potentially active endophytes from roots grown in soil based on 16S rRNA sequencing

Table S10 The relative abundance and taxonomy assignment of the core OTUs shared by seeds and roots at seedling stage based on 16S rRNA sequencing

Table S11 The relative abundance and taxonomy assignment of the core OTUs shared by seeds and roots at booting stage based on 16S rRNA sequencing

Table S12 The relative abundance and taxonomy assignment of the core OTUs shared by seeds and roots at both seedling and booting stages based on 16S rRNA sequencing

Table S13 Taxa differed statistically in the relative abundance in potentially active barley seed and root endophytes based on 16S rRNA sequencing

Table S1 Summary of the relative abundance and taxonomy assignment of the core OTUs in the resident groups of barley seed endophytes

Number of OTUs	Average relative abundance in each cultivar (%)					Taxonomy				
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
6 OTUS	0.48	0.51	0.61	0.48	0.59	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA
4 OTUs	0.29	0.26	0.31	0.25	0.31	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Mycetocola
2 OTUs	0.08	0.07	0.09	0.11	0.11	Actinobacteria	Actinobacteria	Actinomycetales	Sanguibacteraceae	Sanguibacter
2 OTUs	0.03	0.99	0.03	0.04	0.04	Firmicutes	Bacilli	Bacillales	NA	NA
1 OTU	0.00	0.23	0.01	0.01	0.01	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	NA
3 OTUs	0.05	1.59	0.05	0.05	0.04	Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	Exiguobacterium
32 OTUs	5.45	5.96	8.58	4.96	5.58	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus
12 OTUs	0.59	0.47	0.53	0.61	0.7	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Saccharibacillus
1 OTU	0.05	0.05	0.05	0.08	0.07	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Diaphorobacter
2 OTUs	0.04	0.04	0.03	0.05	0.04	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	NA
5 OTUs	0.15	0.12	0.15	0.15	0.15	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium
2 OTUs	0.04	0.04	0.04	0.05	0.05	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Massilia
2 OTUs	0.08	0.04	0.34	0.03	0.06	Proteobacteria	Gammaproteobacteria	NA	NA	NA
74 OTUs	26.6	25.27	25.33	26.12	25.98	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
125 OTUs	52.57	50.58	49.3	52.74	51.92	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
5 OTUs	0.15	0.14	0.09	0.10	0.11	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
8 OTUs	0.54	0.37	0.23	0.23	0.27	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea
1 OTU	0.33	0.3	0.29	0.23	0.25	Proteobacteria	Gammaproteobacteri a	Enterobacteriales	Enterobacteriaceae	Trabulsiella
1 OTU	0.02	0.01	0.11	0.01	0.01	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Xenorhabdus
7 OTUs	0.34	0.33	0.49	0.36	0.34	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	NA
91 OTUs	8.75	8.98	9.13	9.99	9.8	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
18 OTUs	1.77	1.56	1.75	2.07	2.00	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonas

NA: not assigned

Table S2 Summary of the relative abundance and taxonomy assignment of the core OTUs in the potentially active groups of barley seed endophytes

Number of OTUs	Average relative abundance in each cultivar (%)					Taxonomy				
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
4 OTUs	35.68	43.85	7.16	57.00	52.16	Proteobacteria	α -proteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacterium
4 OTUa	11.33	16.27	74.65	4.96	0.85	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus
1 OTU	1.59	0.05	0.01	0.69	0.06	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Saccharibacillus
2 OTUs	1.70	3.17	2.27	1.93	1.76	Proteobacteria	γ - proteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
1 OTU	0.75	1.82	0.94	3.83	0.04	Proteobacteria	γ - proteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
2 OTUs	0.92	1.41	0.26	1.78	1.50	Proteobacteria	γ - proteobacteria	Enterobacteriales	Enterobacteriaceae	NA
3 OTUs	1.30	2.94	5.44	0.99	0.63	Proteobacteria	γ - proteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
1 OTU	0.23	0.34	0.04	0.38	0.14	Proteobacteria	β – proteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia
1 OTU	0.12	0.15	0.01	0.1	0.11	Proteobacteria	β - proteobacteria	Burkholderiales	Comamonadaceae	Delftia
1 OTU	0.09	0.17	0.02	0.14	0.08	Proteobacteria	γ – proteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomona
1 OTU	0.47	0.97	0.19	0.89	1.48	Actinobacteri	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacteriu

NA: not assigned

Table S3 Summary of the relative abundance and taxonomy assignment of the core OTUs in the resident root endophytes in axenic systems

Number of OTUs	Taxonomy					Taxonomy				
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
1 OTU	2.12	12.67	2.12	0.8	2.11	Bacillus	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus
2 OTUs	0.15	0.72	0.15	0.02	0.16	Proteobacteria	Gammaproteobacteria	NA	NA	NA
9 OTUs	0.66	4.32	2.84	35.81	0.46	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
17 OTUs	50.32	21.45	43.36	1.21	47.93	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
1 OTU	0.03	0.07	0.06	0	0.03	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
1 OTU	0.05	0.26	0.06	0.02	0.04	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
8 OTUs	0.75	8.65	2.13	3.39	0.7	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas

NA: not assigned

Table S4 The average abundance and taxonomy assignment of the core OTUs shared by barley resident seed and root endophytes

OTUs	Mean relative abundance(%)		Taxonomy				
	Seeds	Roots	Phylum	Class	Order	Family	Genus
OTU1186	1.89	1.94	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus
OTU1205	1.32	9.64	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU2379	1.93	8.01	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU552376	0.78	6.83	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU7904	0.93	6.80	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU3772	0.50	1.88	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU116	0.27	1.33	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU1204	0.21	1.01	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU4662	0.15	0.82	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU1010113	0.59	0.27	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU5973	0.51	0.24	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU192215	0.09	0.11	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU813217	0.16	0.11	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU3420	0.30	0.04	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU3412	0.20	0.02	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU1042	0.04	0.02	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobacter
OTU922761	0.03	0.03	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia
OTU6718	0.28	0.04	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
OTU776980	7.04	6.69	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU6851	1.12	0.78	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU661	0.43	0.28	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU4408129	0.12	0.26	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU4300	0.03	0.03	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU5512	0.39	0.03	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU817734	0.77	0.53	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU98	1.54	0.44	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU4432796	0.78	0.30	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU7836	0.17	0.19	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU1601	0.68	0.18	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU6526	0.04	0.06	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU2858	0.01	0.03	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU5339	0.01	0.02	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU7107	0.02	0.08	Proteobacteria	Gammaproteobacteria	NA	NA	NA

NA: not assigned

Table S5 The relative abundance and taxonomy assignment of the core OTUs in the potentially active root endophytes

OTU ID	Relative abundance in each cultivar (%)					Taxonomy				
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
OTU 791973	11.91	10.88	10.97	0.004	19.39	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU 578606	0.33	1.69	1.76	8.78	3.16	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU 541859	0.06	0.13	0.64	5.84	0.16	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU 725048	29.89	25.06	36.5	0.02	25.85	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
OTU 554163	0.12	0.09	0.17	0.29	0.07	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA

NA: not assigned

Table S6 The average abundance and taxonomy assignment of the core OTUs shared by the potentially active seed and root endophytes

OTU ID	Mean relative abundance (%)		Taxonomy				
	Seeds	Roots	Phylum	Class	Order	Family	Genus
OTU 725048	2.13	23.46	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella
OTU 791973	0.34	10.63	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU 578606	1.76	3.14	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU 541859	0.15	1.37	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas

Table S7 The relative abundance and taxonomy assignment of the core OTUs in the potentially active root endophytes at seedling stage

Number of OTUs	Mean relative abundance (%)				Taxonomy				
	Alexis	Barke	Salome	Simba	Phylum	Class	Order	Family	Genus
1 OTU	0.01	0.02	0.06	0.14	Actinobacteria	Actinobacteria	Actinomycetales	NA	NA
1 OTU	21.96	0.10	1.99	4.27	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	NA
1 OTU	0.03	0.44	0.18	0.17	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
1 OTU	0.14	0.41	0.03	0.06	Actinobacteria	Actinobacteria	Actinomycetales	Streptosporangiaceae	NA
1 OTU	0.01	0.04	0.08	0.03	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus
1 OTU	0.01	0.79	0.07	0.03	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
1 OTU	0.07	0.06	0.2	0.1	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea
1 OTU	0.69	0.52	5.25	0.84	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
2 OTUs	0.09	0.12	1.47	0.22	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
1 OTU	0.86	0.76	0.2	1.67	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacterium
1 OTU	0.12	0.1	0.16	0.03	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
1 OTU	0.22	0.18	0.04	0.03	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella
1 OTU	0.03	0.12	0.33	0.05	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas
1 OTU	0.01	0.84	0.08	0.03	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax
1 OTU	0.04	0.08	0.04	0.08	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Tepidimonas
1 OTU	0.03	0.11	0.05	0.05	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax
1 OTU	0.02	0.03	0.01	0.01	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia
1 OTU	0.03	0.04	0.04	0.07	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter

NA: not assigned

Table S8 Summary of the relative abundance and taxonomy assignment of the core OTUs in the potentially active root endophytes at booting stage

Number of OTUs	Mean relative abundance in each cultivar (%)					Taxonomy				
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
1 OTU	0.04	0.54	0.65	0.02	0.29	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	NA
1 OTU	0.02	0.5	1.63	0.02	0.04	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA
1 OTU	0.06	0.02	0.13	0.02	0.02	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Agrococcus
5 OTUs	1.85	1.5	5.53	1.27	1.92	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium
1 OTU	0.06	0.03	0.08	0.13	0.05	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium
1 OTU	0.14	0.03	0.29	0.63	0.17	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium
2 OTUs	4.12	0.06	0.67	19.07	1.08	Actinobacteria	Actinobacteria	Actinomycetales	Nocardiaceae	Rhodococcus
1 OTU	0.14	0.05	0.06	0.04	0.09	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
1 OTU	1.4	0.21	0.21	0.46	1.59	Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces
2 OTUs	0.42	2.01	0.27	0.1	0.21	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
1 OTU	0.15	0.05	0.67	0.11	0.39	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	NA
1 OTU	0.18	2.55	0.09	0.07	0.02	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea
1 OTU	0.89	2.71	0.41	0.12	0.19	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
4 OTUs	2.26	1.17	1.46	0.3	1.51	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia
1 OTU	0.06	0.49	0.02	0.11	0.11	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes
1 OTU	0.29	0.15	0.05	0.01	0.06	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter
2 OTUs	0.49	0.1	0.28	0.27	0.37	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
2 OTUs	0.69	0.19	1.04	0.2	0.56	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	NA
5 OTUs	6.18	8.51	3.6	2.63	6.55	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium
1 OTU	0.07	0.06	0.03	0.02	0.02	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Kaistia
2 OTUs	0.65	1.7	0.97	1.55	1.07	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
2 OTUs	4.94	1.24	1.72	2.49	2.22	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella
1 OTU	0.05	0.18	0.01	0.16	0.16	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Azospirillum
1 OTU	0.05	0.13	0.08	0.05	0.04	Proteobacteria	Alphaproteobacteria	Sphingomonadal	Sphingomonadaceae	NA
3 OTUs	0.46	0.43	0.2	0.11	0.05	Proteobacteria	Alphaproteobacteria	Sphingomonadal	Sphingomonadaceae	Sphingomonas
1 OTU	0.11	0.7	0.06	0.08	1.14	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter
1 OTU	1.93	0.52	0.82	0.37	0.34	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax
1 OTU	0.14	0.07	0.68	0.12	0.25	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Polaromonas
2 OTUs	0.53	0.87	6.77	2.89	0.33	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax
1 OTU	0.31	0.08	0.62	0.43	0.46	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	NA
1 OTU	0.54	0.06	0.09	5.15	0.71	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum
1 OTU	0.04	0.03	0.01	0.34	3.71	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA
7 OTUs	8.25	7.07	33.29	12.44	19.98	Proteobacteria	Gammaproteobacteria	Pseudomonadale	Pseudomonadaceae	Pseudomonas
1 OTU	0.11	0.15	0.03	0.45	0.04	Proteobacteria	Gammaproteobacteria	Xanthomonadale	Xanthomonadaceae	Stenotrophomonas

NA: not assigned

Table S9 The relative abundance and taxonomy assignment of the core OTUs shared across all cultivars and both growth stages in the potentially active endophytes from roots grown in soil based on 16S rRNA sequencing

OTU ID	Mean relative abundance (%)		Taxonomy				
	Seedling	Booting	Phylum	Class	Order	Family	Genus
165421	0.21	0.08	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
OTU594	7.08	0.31	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	NA
303643	0.23	0.48	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter
523224	0.11	0.58	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea
826270	1.83	0.86	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
102142	0.1	0.65	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium
136749	0.12	1.65	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella
705063	0.15	0.16	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
806201	0.33	0.15	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
OTU922	0.13	0.1	Proteobacteria	Alphaproteobacteria	Sphingomonadale	Sphingomonadaceae	Sphingomonas
590047	0.24	0.8	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax
819037	0.06	1.36	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax

NA: not assigned

Table S10 The relative abundance and taxonomy assignment of the core OTUs shared by barley seeds and roots at seedling stage based on 16S rRNA sequencing

OTU ID	Mean relative abundance in seeds (%)					Mean relative abundance in roots (%)				Taxonomy				
	Ale	Bar	Mar	Sal	Sim	Ale	Bar	Sal	Sim	Phylum	Class	Order	Family	Genus
165421	0.47	0.97	0.19	0.88	1.49	0.03	0.44	0.18	0.17	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
219107	34.62	42.64	6.89	55.00	50.8	0.86	0.76	0.20	1.67	Proteobacteria	-proteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacterium
705063	0.03	0.04	0.01	0.56	0.05	0.07	0.07	0.41	0.05	Proteobacteria	-proteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
299883	0.23	0.34	0.04	0.38	0.14	0.02	0.03	0.01	0.01	Proteobacteria	-aproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia

Table S11 The relative abundance and taxonomy assignment of the core OTUs shared by barley seeds and roots at booting stage based on 16S rRNA sequencing

OTU ID	Mean relative abundance in seeds (%)					Mean relative abundance in roots at booting stage (%)					Taxonomy				
	Ale	Bar	Mar	Sal	Sim	Ale	Bar	Mar	Sal	Sim	Phylum	Class	Order	Family	Genus
165421	0.47	0.97	0.19	0.88	1.49	0.14	0.05	0.06	0.04	0.09	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
705063	0.03	0.04	0.01	0.56	0.05	0.15	0.02	0.10	0.21	0.30	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium
791973	0.88	0.45	0.03	0.23	0.12	3.99	1.2	1.25	0.84	1.13	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
578606	0.36	2.36	4.93	0.67	0.47	0.22	4.28	18.75	0.13	0.45	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
345540	0.09	0.17	0.02	0.14	0.08	0.11	0.15	0.03	0.45	0.04	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas

Table S12 The relative abundance and taxonomy assignment of the core OTUs shared by barley seeds and roots at both seedling and booting stages based on 16S rRNA sequencing

OTU ID	Mean relative abundance (%)			Taxonomy				
	Seeds	Seedling	Booting	Phylum	Class	Order	Family	Genus
165421	0.801	0.202	0.077	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
705063	0.14	0.168	0.166	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Mesorhizobium

Table S13 Taxa differed statistically in the relative abundance in potentially active seed and root endophytes based on 16S rRNA sequencing

(a) Taxa enriched in roots and their mean relative abundance in seeds and roots

Taxonomy					Mean relative abundance (%)	
Phylum	Class	Order	Family	Genus	Seeds	Roots
Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	Other	0	3.65
Actinobacteria	Actinobacteria	Actinomycetales	Frankiaceae	Actinomycetales	0	0.19
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Cryocola	0.001	0.52
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	0.05	3.13
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	NA	0.22	1.66
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Salinibacterium	0.14	0.23
Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	NA	0.01	1.3
Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	Actinoplanes	0	0.7
Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	Mycobacterium	0.19	0.93
Actinobacteria	Actinobacteria	Actinomycetales	Other	Other	0.02	1.53
Actinobacteria	Actinobacteria	Actinomycetales	Pseudonocardiaceae	Amycolatopsis	0	0.66
Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Other	0	0.14
Actinobacteria	Actinobacteria	Actinomycetales	Streptomycetaceae	Streptomyces	0	7.84
Actinobacteria	Actinobacteria	Actinomycetales	Streptosporangiaceae	NA	0	0.07
Actinobacteria	Thermoleophilia	Solirubrobacterales	NA	NA	0.03	0.44
Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Niastella	0	0.08
Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	0.003	3.13
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Other	0	0.01
Firmicutes	Bacilli	Bacillales	Pasteuriaceae	Pasteuria	0	0.05
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	02d06	0	0.38
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium	0	3.61
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Caulobacter	0.004	0.6
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	NA	0.003	0.3
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Phenylobacterium	0.02	0.12
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea	0.04	0.51
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium	0.43	1.42
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Other	0.01	0.57
Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Devosia	0.04	1.16
Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA	0.01	0.11
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Aminobacter	0.002	0.06
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium	0.11	4.35
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Kaistia	0.05	0.17
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	NA	0.003	0.24
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Other	0.35	0.62
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Rhizobium	0.23	1.28
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella	0.02	1.86
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Sinorhizobium	0.002	0.18
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Other	0	0.11
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	NA	0	0.13
Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Other	0.01	0.22
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	0.001	0.25
Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Other	0	0.32
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax	0.003	0.56
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Delftia	0.1	0.45
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Hydrogenophaga	0	0.06
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Leptothrix	0.001	0.25
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Other	0.001	0.15
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Roseateles	0	0.23
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax	0.01	0.04
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Tepidimonas	0	0.04
Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Variovorax	0.11	1.42
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Herbaspirillum	0.001	0.87
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Janthinobacterium	0.11	1.27
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Cellvibrio	0	0.1
Proteobacteria	Gammaproteobacteria	Other	Other	Other	0	0.11
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae	NA	0.24	1.1
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Luteibacter	0.002	0.13
Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Rhodanobacter	0	0.42

(b) Taxa depleted in roots and their mean relative abundance in seeds and roots

Taxonomy					Mean relative abundance (%)	
Phylum	Class	Order	Family	Genus	Seeds	Roots
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Curtobacterium	1.03	0.15
Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Frigoribacterium	0.31	0
Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	0.83	0.14
Actinobacteria	Actinobacteria	Actinomycetales	Sanguibacteraceae	Sanguibacter	1.69	0.05
Firmicutes	Bacilli	Bacillales	[Exiguobacteraceae]	Exiguobacterium	1.74	0
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus	23.22	0.78
Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Saccharibacillus	0.58	0.02
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Other	0.17	0
Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacterium	39.3	0.47
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	NA	0.35	0.25
Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Ralstonia	0.23	0.02
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia	9.1	1.11
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	0.04	0.01
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Trabulsiella	2.61	0.08

NA: not assigned

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Appendix

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ORIGINAL RESEARCH
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Dominant Groups of Potentially Active Bacteria Shared by Barley Seeds become Less Abundant in Root Associated Microbiome

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Endophytes are microorganisms colonizing plant internal tissues. They are ubiquitously associated with plants and play an important role in plant growth and health. In this work, we grew five modern cultivars of barley in axenic systems using sterile sand mixture as well as in greenhouse with natural soil. We characterized the potentially active microbial communities associated with seeds and roots using rRNA based amplicon sequencing. The seeds of the different cultivars share a great part of their microbiome, as we observed a predominance of a few bacterial OTUs assigned to *Phyllobacterium*, *Paenibacillus*, and *Trabusiella*. Seed endophytes, particularly members of the Enterobacteriacea and Paenibacillaceae, were important members of root endophytes in axenic systems, where there were no external microbes. However, when plants were grown in soil, seed endophytes became less abundant in root associated microbiome. We observed a clear enrichment of Actinobacteriacea and Rhizobiaceae, indicating a strong influence of the soil bacterial communities on the composition of the root microbiome. Two OTUs assigned to Phyllobacteriaceae were found in all seeds and root samples growing in soil, indicating a relationship between seed-borne and root associated microbiome in barley. Even though the role of endophytic bacteria remains to be clarified, it is known that many members of the genera detected in our study produce phytohormones, shape seedling exudate profile and may play an important role in germination and establishment of the seedlings.

Keywords: ***Hordeum vulgare*** **L., root endophytes, seed microbiome, 16S rRNA barcoding**

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INTRODUCTION

Microorganisms living in close association with plants have significant impact on plant growth and health. Hence, the plant associated microbiome is often referred to as the "second genome" of plants (Berendsen et al., 2012). A special role has been assigned to those communities living inside plant organs for all or part of their lifetime, termed as endophytes (Hardoim et al., 2015). Due to their intimate association with plant tissues (Han et al., 2016), they impact the development of the host significantly (Berg et al., 2016; Kaul et al., 2016).

Thus, not surprisingly, a substantial amount of work has been done in the past to characterize the structure and function of root endophytes (Bulgarelli et al., 2012, 2015; Lundberg et al., 2012;

Peiffer et al., 2013; Edwards et al., 2015). However, most current studies focused on the presence of resident communities. Yet it is important to note that some "opportunistic endophytes" (Hardoim et al., 2008) or "passenger endophytes" (de Almeida et al., 2009) may enter the plant endosphere just by chance. Their functional contributions to the community could be limited, if they are only transient or dormant. A previous study has shown that the active endophytic groups were less complex than the resident community (Reiter et al., 2003). Therefore, more study of the active endophytic community is needed toward a better understanding of plant and endophyte interaction.

It was demonstrated that seed-borne endophytes are able to persist in the seedlings as almost all genera isolated from seeds were also recovered from bean roots (Lopez-Lopez et al., 2010). Hardoim et al. (2012) showed that seed endophytes of rice are important founders of bacteria colonizing the root interior using a fingerprinting method. Bacteria from the external environment, basically soil, will also colonize plants, leading to shifts in bacterial community structure during root development (Kristin and Miranda, 2013). However, the dynamics of seed-borne endophytes during seed germination and root development are still not clear.

A recent study indicated that seed associated microorganisms may release seed dormancy through production of cytokinins (Goggin et al., 2015). Puente et al. (2009) demonstrated that bacterial endophytes from cactus seeds could improve the establishment of seedlings on barren rocks. Seedling development was stopped when disinfecting cactus seeds with antibiotics. However, although the seed associated microbiome obviously strongly impacts plant growth and health, little is known about the structure and regulators of seed associated microbiome.

In this study, we focused on the potentially active bacterial community. We investigated (a) plant cultivar dependent effects of the seed microbiome (b) the role of the seed microbiome as "first inoculum" of root endophytes and (c) the stability of this "first inoculum" during plant development. We used different cultivars of barley as a model and performed a greenhouse experiment using soil as well as experiments in axenic systems using sterile sand mixture. Bacterial communities were analyzed from surface sterilized seeds and roots using barcode sequencing based on rRNA. We postulate (a) cultivar dependent differences in the seed microbiome structure are low and (b) that the seed microbiome will make a significant part of the root microbiome at early plant growth stages, being further substituted by bacterial populations present in the rhizosphere.

MATERIALS AND METHODS

Seeds Surface Sterilization

In the frame of this study, we used barley cultivars Alexis, Barke, Marthe, Salome, and Simba. Alexis and Barke were obtained from Saatzucht Breun GmbH & Co. KG (Herzogenaurach, Germany), while Marthe, Salome, and Simba were supplied by Nordsaat Saatzucht GmbH (Langenstein, Germany). Surface sterilization of seeds was performed using 70% ethanol for 5 min and 2% NaClO for 20 min. This method was selected because a microscopic comparison showed that this method is more efficient in removing surface microbes than commonly used ultrasonication and shaking (Reinhold-Hurek et al., 2015). Detailed procedures of the surface sterilization have been described previously (Kutter et al., 2006). The success of the surface sterility for seeds was checked by FISH using Eub-335-I, Eub-335-II, and Eub-338-III (Metabion, Germany) as described elsewhere (Spohn et al., 2015) and plating on R2A agar plates.

RNA and DNA Co-extraction from Seeds

After plating on R2A agar plates for 24 h at 23°C in dark, the imbibed seeds were used for nucleic acid extraction. Each sample was composed of six seeds, which were grounded using liquid nitrogen with a mortar and pestle. 0.1 g from the seed powder was used for a coextraction of DNA and RNA using Griffiths' protocol (Griffiths et al., 2000). Extraction was performed for each cultivar in five replicates (each consisting of six seeds). Water served as a negative control and was used for extraction of nucleic acids in a parallel approach.

DNA/RNA co-extracts were digested with DNase Max™ Kit (MoBio, United States) to obtain pure RNA. Complete DNA digestion was checked and confirmed with real time quantitative PCR for 16S rRNA genes using the primer set 968F/1401R. The resulting purified RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States). The other aliquot was left untreated and is from here on referred to as DNA. DNA and cDNA samples were stored at −80°C until further analysis.

Barley Cultivation

For barley cultivation, surface sterilized seeds were germinated on a wet paper in Petri dishes in the dark for 3 days at 30°C. In this work, we used two systems to investigate the impact of seed-borne endophytes on the composition of the root associated microbiome. To study the root endophytes originating from seeds, we created axenic systems where there are no external microbes. We also used soil based systems, which resemble natural conditions, to investigate to what extent seed-borne endophytes can persist in roots when microbes from the rhizosphere also colonize the root interior.

Axenic systems were made using sterile beakers (250 ml), sterile glass beads (185 g) and 45 ml sterile MS media (Duchefa Biochemie bv, The Netherlands). Six germinated seeds were put in the glass beads and covered with another sterile beaker. The complete system was then sealed with Parafilm. Five replicates (each consisting of six seeds) were used per cultivar. Plants were grown in a climate chamber under controlled conditions (23°C/14 h, 15°C/10 h, and 65% humidity).

For "soil based systems," germinated seeds were sown in pots filled with sandy soil. The soil was collected from the top layer from an arable field in Scheyern Research Farm (Scheyern, Germany) in July, 2014 and was sieved using a 2 mm mesh. The pots were 13 cm high, with the top square 13 × 13 cm and 9.6 × 9.6 cm at the bottom. The soil was filled to a depth of 10 cm in the pot. Every pot contained one well-germinated seed.

For each cultivar four replicates were prepared. The plants were grown in a greenhouse under controlled conditions with 12 h light at 20°C and 12 h dark at 16°C. The plants were watered twice a week to obtain a water content of 60% of the maximal water holding capacity.

Roots Sampling and Surface Sterilization

We used Zadoks decimal code (Zadoks et al., 1974) for the growth stages scale and determined our sampling time accordingly. Barley plants growing in axenic systems were sampled 8 days (seedling growth, Z13) after sowing the seeds. Plants growing in the greenhouse were harvested at two time points, 2 weeks after planting (seedling growth, Z13) and 10 weeks after planting (booting, Z41). Before surface sterilization the remaining sand/soil from the roots was removed by shaking and washing in water.

Roots were sterilized like described above for seeds, washed five times with sterile water and shock frozen using liquid nitrogen. Root samples were grounded to powder using the TissueLyzer II (Qiagen, Germany) according to the manufacturer's instructions. RNA extraction, reverse transcription and sample handling was done as described above.

Library Preparation and Sequencing

In the frame of this project, primer pair S-D-Bact-0008-a-S-16 (Muyzer et al., 1993) and S-D-Bact-0343-a-A-15 (Alm et al., 1996) was used (Klindworth et al., 2013). As preliminary data indicated a huge co-amplification of plastids when DNA was used as a target, we used RNA in this study, as plastid content in rRNA is low (Supplementary Figure S1). To compare the resident and active community, we also performed DNA amplification using the primer 338F/789R, which was reported to exclude chloroplast amplification (Dorn-In et al., 2015). Our data indicated a higher number of genera in 16S rRNA sequences when primer pair S-D-Bact-0008-a-S-16 and S-D-Bact-0343-a-A-15 was used to amplify the obtained cDNA compared to DNA amplification using 338F/789R. It also confirmed a strong bias of the primer 338F/789R, which was mainly a result of the predominance of Enterobacteriaceae, whereas the percentage of Enterobacteriaceae was much lower in the rRNA samples (Supplementary Figure S2). Therefore, we chose the primer pair S-D-Bact-0008-a-S-16 and S-D-Bact-0343-a-A-15 for the analysis of the active fraction of the community.

The PCR conditions were the following: 98°C for 5 min, followed by 30 cycles each at 98°C for 10 s, 60°C for 30 s and 72°C for 30 s, followed by 72°C for 5 min. Triplicate amplicons were pooled and purified using Agencourt AMPure XP kit (Beckman Coulter, United States). DNA quantity was assessed with the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, United States). Nextera XT Index Kit v2 (Illumina, United States) was used for amplicon indexing. Reactions were kept at 98°C for 5 min, followed by eight cycles at 98°C for 10 s, 55°C for 30 s and 72°C for 30 s, with a final extension step of 10 min at 72°C. All amplicons were purified and quantified as described above. The purified amplicons were then pooled in 4 nM concentrations and sequenced on Illumina Miseq platform (Illumina, United States). The obtained sequences were deposited under the accession number SRP102191 in the SRA.

Data Analysis

The sequencing analysis was performed with the software QIIME (version 1.9.0) (Caporaso et al., 2010). Adaptors and primers were removed using AdapterRemoval (Lindgreen, 2012). Phix contamination was removed using the program Deconseq (Schmieder and Edwards, 2011). Reads were merged and filtered by size (according to primer set) and quality (Phred quality score > 2). The sequences were then clustered into operational taxonomic units (OTUs) using an open reference strategy based on 97% identity with GreenGenes Database (13_5 release) (DeSantis et al., 2006) as reference. Taxonomy was assigned with RDP classifier (Wang et al., 2007) retrained with GreenGenes 16S rRNA database (13_5 release). OTUs assigned to chloroplast were filtered out.

The statistical analysis was also performed using QIIME (version 1.9.0). Plots were generated with R (version 3.2.1) using packages vegan, plyr, beanplot, ggplot, and vcd.

RESULTS

Sequencing Summary

A total of 7,838,588 raw sequences were obtained. The number of reads per sample ranged from 11,901 to 199,129. After adaptor, primer and chimera removal as well as length and quality filtering, 6,547,064 high-quality reads were clustered at 97% sequence identity. OTUs assigned to chloroplast were discarded, resulting in 5,816,127 remaining reads. Low abundant OTUs (less than 0.005%) were filtered out, resulting in 851 OTUs. To compare the diversity in different samples, we rarefied the data to 11,390 reads per sample for comparison. Rarefaction curves indicated that the sequencing depth is sufficient to capture the microbial diversity (Supplementary Figure S3).

Active Bacterial Groups in Seeds

For the active seed associated microbiome, we identified 137 genera from 83 families of 10 different phyla based on our molecular barcoding approach (**Figure 1A**). To investigate the genotype effect on the active seed associated microbiome, we carried out principal coordinate analysis (PCoA) both based on weighted and unweighted Unifrac distance metrics (**Figures 2A,B**). Permutational multivariate analysis of variance using distance matrices (ADONIS) showed significant differences between active bacterial communities across cultivars (weighted Unifrac, $p = 0.001$, $R^2 = 0.81$; unweighted Unifrac, $p = 0.001$, $R^2 = 0.31$). We found two OTUs which differed in frequencies across all cultivars using Kruskal–Wallis test (Bonferroni corrected p-value < 0.05). These two OTUs were assigned to *Paenibacillus* and *Pseudomonas*.

Despite of these differences, we observed a shared set of associated bacteria. 21 core OTUs were found in all cultivars, which were assigned to Phyllobacteriaceae (four OTUs), Paenibacillaceae (five OTUs), Enterobacteriaceae (five OTUs), Pseudomonadaceae (three OTUs), Oxalobacteraceae

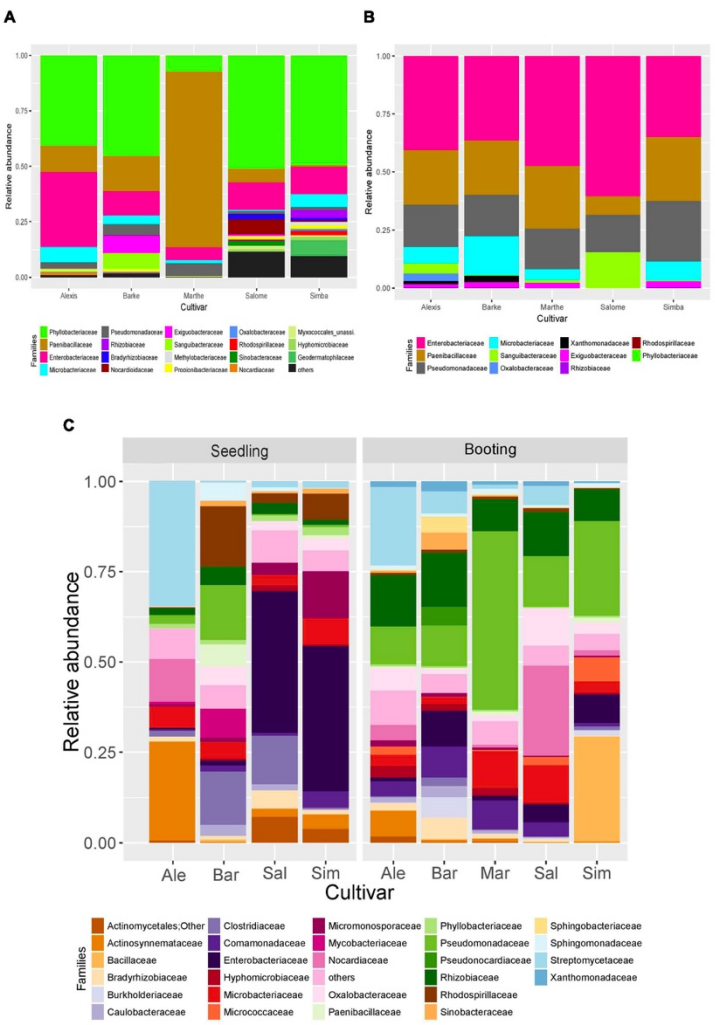

FIGURE 1 | The structure of bacterial communities associated with **(A)** seeds **(B)** roots growing in axenic systems and **(C)** roots growing in greenhouse at family level. Ale, Alexis; Bar, Barke; Sal, Salome; Mar, Marthe; Sim, Simba (n = 3–5). OTUs with abundance less than 0.005% were filtered out.

(one OTU), Comamonadaceae (one OTU), Xanthomonadaceae (one OTU), and Propionibacteriaceae (one OTU) (**Table 1**). These core OTUs represented, in total, more than 50% of all reads. Notably, five OTUs assigned to Phyllobacteriaceae, Paenibacillaceae, Pseudomonadaceae, and Enterobacteriaceae, respectively contributed to most of the reads, while others had relative abundances of less than 1%.

Active Bacterial Groups in Roots

The active bacteria associated with roots growing in axenic systems differed significantly in α diversity across cultivars ($p < 0.05$) (Supplementary Figure S4). Differences in ß diversity were also detected in both weighted (ADONIS, $p = 0.002$, $R^2 = 0.43$) and unweighted (ADONIS, $p = 0.001$, $R^2 = 0.66$) Unifrac distance metrics (**Figures 2C,D**). Only five core OTUs

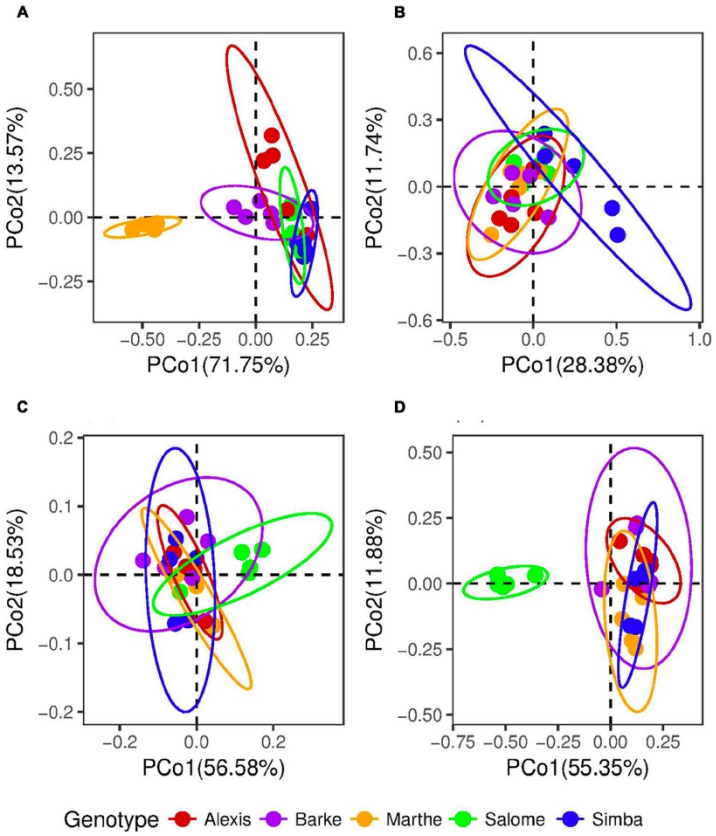

FIGURE 2 | Principal coordinate analysis (PCoA) plot to investigate the differnce of microbiome associated with seeds and roots (n = 3–5). **(A)** Seed samples based on weighted Unifrac and **(B)** unweighted Unifrac distance metrics; **(C)** root samples from axenic systems based on weighted Unifrac and **(D)** unweighted Unifrac distance metrics. The lines of ellipses show 95% confidence intervals.

were found, which were assigned to Enterobacteriaceae and Pseudomonadaceae (**Table 2** and **Figure 1B**). Interestingly, these families are also the most abundant families in the seed associated microbiome.

We further analyzed the active groups associated with roots growing in the soil (**Figure 1C**). We also investigated the influences of genotype and growth stage. Statistical analysis (ADONIS) using weighted Unifrac distances, revealed both genotype and growth stage dependent impacts on barley endophytes (genotype, $p < 0.05$, $R^2 = 0.23$; growth stage, $p < 0.05$, $R^2 = 0.10$) (**Figure 3A**).

However, when unweighted Unifrac metrics were used, the genotype effects were not significant ($p > 0.05$). Only the growth stage accounted for the variation between microbial communities significantly ($p = 0.001$, $R^2 = 0.124$). Consistently, clustering patterns were observed only by growth stages in the ordination plot of PCoA (**Figure 3B**), implying that the plants' developmental stage is the main driving factor in shaping the root associated bacterial community.

To gain insights into the richness of barley root microbiota, we compared the number of observed OTUs and the Chao1 index of the community retrieved from seedling (2 weeks) and booting stage (10 weeks) (Supplementary Figure S5). Endophytes at the booting stage were significantly more diverse, resulting in a higher Chao1 index (t-test, $p = 0.002$).

We found 16 core OTUs at seedling stage and 67 at booting stage (Datasheet S1). Although there was a large overlap between the OTUs at two growth stages, only 10 OTUs were common

TABLE 1 | The relative abundance and taxonomy assignment of core OTUs in seed associated microbiome.

OTU ID	Relative abundance in each genotype (%)					Taxonomy					
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
OTU219107	34.77	42.63	6.91	54.97	50.67	Proteobacteria	α-Proteobacteria	Rhizobiales	Phyllobacteriaceae	*Phyllobacterium*
OTU1095	0.74	0.99	0.21	1.22	1.20	Proteobacteria	α-Proteobacteria	Rhizobiales	Phyllobacteriaceae	*Phyllobacterium*
OTU431921	0.14	0.17	0.03	0.26	0.24	Proteobacteria	α-Proteobacteria	Rhizobiales	Phyllobacteriaceae	*Phyllobacterium*
OTU705063	0.03	0.06	0.01	0.55	0.05	Proteobacteria	α-Proteobacteria	Rhizobiales	Phyllobacteriaceae	*Phyllobacterium*
OTU101	9.21	10.76	59.86	3.08	0.68	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*
OTU415	1.44	2.74	7.53	0.66	0.13	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*
OTU537	0.36	0.65	4.05	0.94	0.02	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*
OTU863	0.32	2.12	3.21	0.28	0.02	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Paenibacillus*
OTU1508	1.59	0.05	0.01	0.69	0.06	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	*Saccharibacillus*
OTU725048	1.68	3.13	2.26	1.86	1.71	Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Trabulsiella*
OTU1109844	0.02	0.04	0.01	0.07	0.05	Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Trabulsiella*
OTU667	0.75	1.82	0.94	3.83	0.04	Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	*Erwinia*
OTU289261	0.76	1.21	0.21	1.58	1.35	Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	NA (not assigned)
OTU541119	0.16	0.20	0.05	0.20	0.15	Proteobacteria	γ-Proteobacteria	Enterobacteriales	Enterobacteriaceae	NA (not assigned)
OTU578606	0.36	2.36	4.96	0.67	0.47	Proteobacteria	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU791973	0.88	0.45	0.03	0.23	0.12	Proteobacteria	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU541859	0.06	0.13	0.45	0.09	0.04	Proteobacteria	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU299883	0.23	0.34	0.04	0.38	0.14	Proteobacteria	β-Proteobacteria	Burkholderiales	Oxalobacteraceae	*Ralstonia*
OTU264546	0.12	0.15	0.01	0.10	0.11	Proteobacteria	β-Proteobacteria	Burkholderiales	Comamonadaceae	*Delftia*
OTU345540	0.09	0.17	0.02	0.14	0.08	Proteobacteria	γ-Proteobacteria	Xanthomonadales	Xanthomonadaceae	*Stenotrophomonas*
OTU165421	0.47	0.97	0.19	0.89	1.48	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	*Propionibacterium*

TABLE 2 | The relative abundance and taxonomy assignment of core OTUs in microbiome associated with seedlings growing in axenic systems.

OTU ID	Relative abundance in each genotype (%)					Taxonomy				
	Alexis	Barke	Marthe	Salome	Simba	Phylum	Class	Order	Family	Genus
OTU 791973	11.91	10.88	10.97	0.004	19.39	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU 578606	0.33	1.69	1.76	8.78	3.16	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU 541859	0.06	0.13	0.64	5.84	0.16	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*
OTU 725048	29.89	25.06	36.50	0.02	25.85	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA (not assigned)
OTU 554163	0.12	0.09	0.17	0.29	0.07	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA (not assigned)

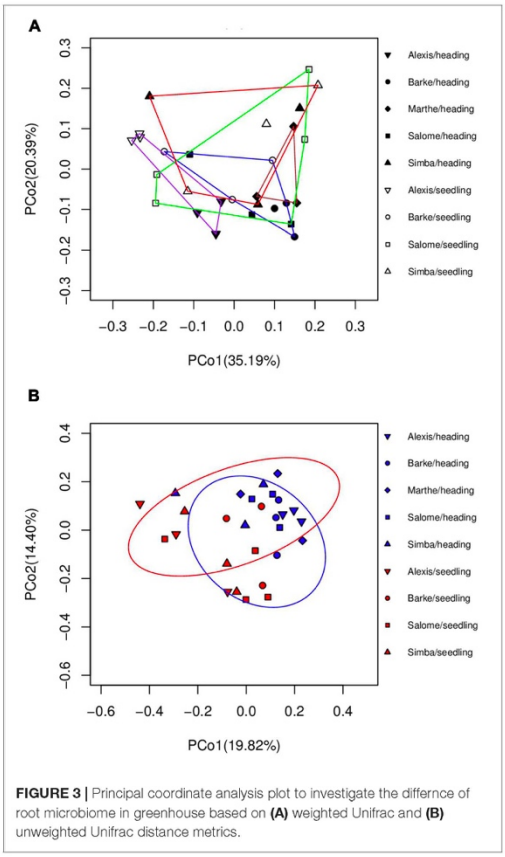

FIGURE 3 | Principal coordinate analysis plot to investigate the differnce of root microbiome in greenhouse based on **(A)** weighted Unifrac and **(B)** unweighted Unifrac distance metrics.

across all cultivars and growth stages in the root associated microbiome, which were assigned to Bradyrhizobiaceae (two OTUs), Comamonadaceae (two OTUs), Phyllobacteriaceae (two OTUs), Actinosynnemataceae (one OTU), Propionibacteriaceae (one OTU), Caulobacteraceae (one OTU), and Rhizobiaceae (one OTU) (**Table 3**). In general, these OTUs were not abundant, most of which had less than 1% of the total reads.

Comparing Seed Microbiome and Root Microbiome

In axenic systems, 18 OTUs were only detected in the roots from plants but not in the seeds. However, at genus level, one genus detected in the roots (*Xanthomonas*) was not found in the seeds. A genotype effect was observed for both seed and root associated microbiomes. This pattern changed when the seeds developed into plants in axenic systems. Marthe had the most divergent seed microbiome while Salome showed the biggest difference in the

TABLE 3 | The relative abundance and taxonomy assignment of core OTUs shared across all cultivars and growth stages in microbiome associated with roots growing in greenhouse.

OTU ID	Relative abundance (%)								Taxonomy			
	2 weeks (seedling statage, Z13)				10 weeks (booting stag, Z41)				Phylum	Class	Order	Family	Genus
	Ale	Bar	Sal	Sim	Ale	Bar	Mar	Sal	Sim				
OTU15	21.23	0.10	1.85	4.12	0.04	0.34	0.62	0.02	0.35	Actinobacteria	Actinobacteria	Actinomycetales	Actinosynnemataceae	NA (not assigned)
OTU165421	0.03	0.43	0.18	0.16	0.14	0.16	0.06	0.04	0.10	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	*Propionibacterium*
OTU303643	0.01	0.82	0.25	0.04	0.19	0.21	0.14	0.10	0.08	Proteobacteria	α-Proteobacteria	Caulobacterales	Caulobacteraceae	NA (not assigned)
OTU826270	0.73	0.54	5.38	0.88	0.92	0.65	0.45	0.12	0.15	Proteobacteria	α-Proteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bradyrhizobium*
OTU523224	0.05	0.05	0.16	0.09	0.17	0.23	0.10	0.07	0.02	Proteobacteria	α-Proteobacteria	Rhizobiales	Bradyrhizobiaceae	NA (not assigned)
OTU102142	0.13	0.11	0.11	0.01	0.41	0.16	0.82	1.98	1.02	Proteobacteria	α-Proteobacteria	Rhizobiales	Rhizobiaceae	NA (not assigned)
OTU705063	0.07	0.07	0.42	0.06	0.15	0.15	0.09	0.22	0.28	Proteobacteria	α-Proteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*
OTU806201	0.02	0.06	1.09	0.18	0.34	0.21	0.20	0.07	0.04	Proteobacteria	α-Proteobacteria	Rhizobiales	Phyllobacteriaceae	*Mesorhizobium*
OTU819037	0.04	0.11	0.04	0.06	0.33	0.67	4.10	1.89	0.18	Proteobacteria	β-Proteobacteria	Burkholderiales	Comamonadaceae	*Variovorax*
OTU590047	0.01	0.84	0.07	0.03	1.90	0.48	0.84	0.36	0.20	Proteobacteria	β-Proteobacteria	Burkholderiales	Comamonadaceae	NA (not assigned)

composition of the root associated communities compared to the other cultivars (**Figures 2A,D**).

All root endophytes shared OTUs with the seed associated microbiome, regardless of their growing conditions. However, plants growing in soil shared fewer OTUs with seeds compared to plants growing in axenic systems (**Figure 4A**), indicating a strong influence of soil microbiota. For plants grown in soil, root endophytes shared OTUs with the seed microbiome, in both seedling and booting stage. But more OTUs were found to overlap between the two growth stages than between the root and seed microbiome (**Figure 4B**).

The heatmap further illustrates the dynamics of bacterial communities (**Figure 5**). Enterobacteriaceae were abundant in both seed microbiome and root microbiome in axenic systems.

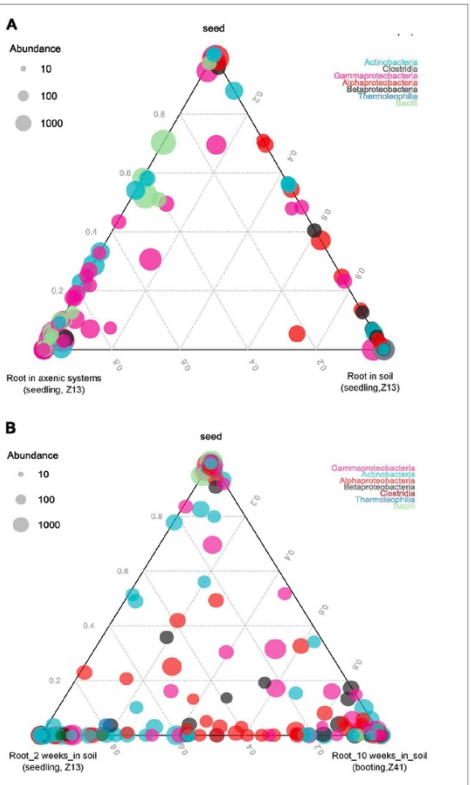

FIGURE 4 | Ternary plot of OTUs showing the distribution of OTUs in different sample categories. **(A)** Seeds and root seedlings (Z13) grown in axenic systems and soil. **(B)** Seeds and roots grown in soil at seedling (Z13) and booting (Z41) stage. Each corner of the triangle represents a sort of sample. The size of plotted dots corresponds to the abundance of the OTUs. The dashed grid lines inside the plot indicate the contribution of each sample type (n = 3–5).

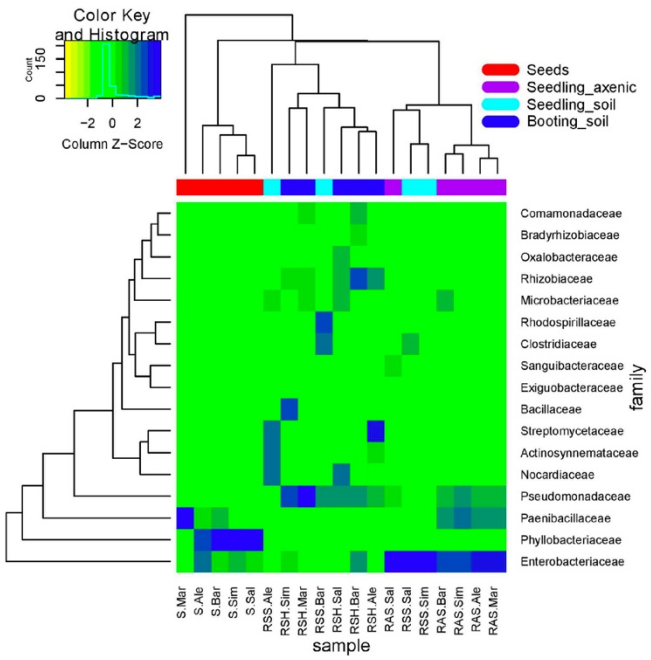

FIGURE 5 | Heat map of the relative abundance of the most abundant families present in seed and root associated microbiome. S, seed; RAS, roots growing in axenic systems (seedling, Z13); RSS, roots growing in soil (seedling, Z13); RSH, roots growing in soil (booting, Z41). Ale, Alexis; Bar, Barke; Sal, Salome; Mar, Marthe; Sim, Simba.

In plants grown in soil, the abundance of Enterobacteriaceae varied across cultivars. Barke and Simba showed a higher abundance of Enterobacteriaceae at the seedling stage (Z13) while Salome and Simba showed a higher abundance at the booting stage (Z41). Phyllobacteriaceae, which was the most abundant family in the seed microbiome, decreased dramatically to less than 2% in the root associated microbiome. In contrast, Pseudomonadaceae were largely enriched in the root associated microbiota. Similarly, an enrichment of Rhizobiaceae was also observed in the root microbiome, but only when plants were grown in soil. Streptomycetaceae, detected in low abundance in seeds and not found in roots growing in axenic systems, appeared to be abundant in roots growing in soil. On the contrary, Paenibacillaceae, highly abundant in both seeds and roots growing in axenic systems, decreased to negligible percentage in roots growing in soil.

DISCUSSION

Seed Associated Microbiome

In this work, we investigated modern commercially available barley cultivars. A significant cultivar effect was observed in the seed associated microbiome. This result was unexpected as we assumed that bacteria colonizing the seed interior are subjected to similar selective pressure and, hence, would not significantly differ between the cultivars. In fact, many studies have shown that the plant cultivar is less relevant for the composition of bacterial communities, whereas the plant compartment plays a major role (Bulgarelli et al., 2012). However, they only analyzed the resident bacteria, while our work studied the potentially active part of the community. The influence of the plant genotype is probably stronger on the potentially active endophytes than on the total community. We also consider that the differences observed in our analysis were driven by the extremely high abundance of *Paenibacillus* sequences in the libraries obtained from Marthe, which were not found in other cultivars.

We observed a dominance of a few bacterial OTUs assigned to *Phyllobacterium* (OTU219107), *Paenibacillus* (OTU101), and *Trabusiella* (OTU725048) in the seeds of the five investigated cultivars. *Phyllobacterium* has been described as a plant-associated genus and was isolated from the rhizosphere, root and nodules from different plant species (Mantelin et al., 2006). It was also shown to be vertically transmitted in *Phaseolus vulgaris* (Lopez-Lopez et al., 2010). Although their role in seeds was not

yet investigated, *Phyllobacterium* was shown to promote root growth in *Brassica napus* and *Arabidopsis thaliana* (Bertrand et al., 2001; Contesto et al., 2010; Kechid et al., 2013).

Some *Paenibacillus* strains produce cytokinins (Timmusk and Wagner, 1999), which are directly involved in seed germination (Kumar et al., 2014). Goggin et al. (2015) showed that the reduction of the density of endophytic populations, e.g., by heating, made seeds unable to lose dormancy. They postulated that this was caused by a decrease in the concentration of cytokinins of bacterial origin. In fact, the inoculation of *A. thaliana* with a *Paenibacillus polymyxa* strain reduced the germination time (Kefela et al., 2015).

Moreover, bacteria were shown to alleviate reactive oxygen species (ROS) stress, allowing quinoa seeds to germinate even under hostile environmental conditions (Pitzschke, 2016). It is known that ROS, namely hydrogen peroxide, induces a mitogen-activated protein kinases (MAPKs) dependent decrease of abscisic acid content, a hormone known to inhibit germination (Barba-Espin et al., 2011). H_2O_2 also acts as a priming factor that promotes changes on seed proteome, which may relieve seeds from dormancy (Oracz et al., 2008). Nevertheless, at higher concentrations, ROS may cause tissue damage. Therefore, for germination to occur, it is necessary that ROS are kept at a certain level, the so-called "oxidative window." Although not shown for seeds, bacteria from the genus *Paenibacillus* were shown to reduce oxidative stress in legume nodules (Rodrigues et al., 2013).

Trabusiella was also shown to contribute with a great part to the seed microbiome in barley. The two species described within the genus *Trabusiella* are not plant associated bacteria. However, *Trabusiella* OTUs and other genera within the family Enterobacteriaceae were also found in high abundance in seeds from Agave and many other plant species (Truyens et al., 2015; Coleman-Derr et al., 2016). It was postulated that seed associated Enterobacteriaceae reduce the concentration of seed exudates that trigger the sporulation of fungal pathogens, such as *Phytum ultimatum* (Hood et al., 1998). Proteome analyses showed that during germination barley seeds synthetize and secrete a range of protease inhibitors, probably for the neutralization of fungal exoenzymes (Sultan et al., 2016). Vertical transmission of bacteria that reduce the pathogen sporulation may be another mechanism by which barley plants control infection.

Root Associated Microbiome

In this work, we used two systems to grow barley: axenic systems with sterile sand mixture and greenhouse systems with natural soil.

We observed significant differences on the composition of the microbiome detected in roots of the five cultivars growing in axenic systems. Compared to seeds, we noted a shift in the taxonomical composition. *Phyllobacterium*, *Paenibacillus*, both highly abundant in the seeds, were less numerous in the axenic roots. On the other hand, bacteria belonging to the genera *Pseudomonas* and *Trabusiella* were found largely enriched in root tissue. Two major OTUs, OTU 791973 (*Pseudomonas*) and OTU 725048 (*Trabusiella*), were found in all root and seeds samples. Many strains of these two families were reported to promote plant growth, and were frequently described to be found in roots as well (Bulgarelli et al., 2013; Cope-Selby et al., 2016).

In contrast, cultivar dependent effects were less pronounced in roots growing in soil, and were only significant when calculating the distance between samples using weighted Unifrac metrics. Our results indicate that the divergence of root microbiota across genotypes is only quantitative. The variation between the genotypes was manifested in the abundance of many OTUs from diverse taxa (Streptomycetaceae, Comamonadaceae, Rhizobiaceae, and Nocardiaceae), rather than by the presence/absence of single OTUs in the given genotypes.

These findings are in accordance with a recent study comparing the resident root microbiota of wild and domesticated barley, where a small but significant host genotype effect on the basis of abundance was reported (Bulgarelli et al., 2015). We suppose that the genetic variation across our genotypes is smaller than that in the above study of Bulgarelli et al. (2015) which compared wild and domesticated barley. Therefore, less variation of the associated microbiome is expected. Yet we still observed a significant impact of the plant cultivar, though only quantitatively, indicating that host genotype is an important filter for the active communities inside plants.

Interestingly, OTUs found in the roots of all plants grown in arable soil were in low abundance and differed from those detected in the axenic systems. The different cultivars grown in the same soil were colonized by bacteria belonging to same taxa, but not exactly the same OTUs. This might be a reflection of the great diversity and functional redundancy found in soils. Furthermore, we observed an enrichment of Actinobacteria in roots of plants grown in soil. Actinobacteria are known to produce a number of secondary metabolites that may hamper the growth of other bacteria, including plant pathogens (Palaniyandi et al., 2013). They were also shown to be enriched in the endophytic compartments of *A. thaliana* (Lundberg et al., 2012). Nevertheless, members of the family Pseudomonadaceae were the only bacteria found in high abundance in root tissue independent from growth condition or plant development stage, suggesting a sturdy association of *Pseudomonas* sp. with barley roots.

CONCLUSION

In this study, we characterized active bacterial communities associated with seeds and roots from five commercially available barley cultivars. We found that the genotype is a significant driving factor in shaping the seed associated microbiome. When plants were grown in soil, the developmental stage was found to have a more pronounced impact on the active community composition, whereas the genotype effect was only quantitative. A conserved set of core OTUs was identified, which comprises stable community members belonging to 12 families including Phyllobacteriaceae, Enterobacteriaceae, Pseudomonadaceae, and Propionibacteriaceae. Seed endophytes were an important inoculum for bacterial communities in the roots in early growth stages. Yet, we observed a large shift when the roots develop from seedling to booting stage in soil. Two OTUs assigned to *Phyllobacterium* were found in all

seeds and root samples growing in soil, indicating a relationship between seed-borne and root associated microbiome in barley.

Thus, future studies should be more related to the functions of the seed and root associated microbiome, to clarify their role for plant development and health. Other parts of the microbiome, e.g., fungi, should also be assessed in the future to get an overall overview on the plant associated microbiome.

AUTHOR CONTRIBUTIONS

LY: this author contributed with the experimental design, laboratory work, data analyses, result discussion and text writing. JD: this author participated of the experimental design, laboratory work and data analyses. AS: this author participated of the data analyses and manuscript writing. PS: this author contributed with data analyses, results discussion and manuscript writing. MS: this author contributed with data analyses, results discussion and manuscript writing. VR: this author contributed with the experimental design, data analyses, results discussion and text writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01005/full#supplementary-material

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