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Investigation of plant genetic-microbiome interactions in different plant species

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Abstract

The rhizosphere, i.e. the soil surrounding the plant roots, and endosphere, i.e. the microbial communities within the plant organs harbors microbes known to influence root and plant physiological processes. An important question is to what extent plant species, genotypes and environmental conditions affect bacterial and fungal communities.

The objectives of the first research study were to unravel and compare the rhizospheric microbiota of grape in two independent vineyards using 16S and ITS amplicon sequencing, evaluate location and varietal effects, and test the correlation between bioavailable copper levels and other soil parameters with microbiota composition and diversity. Our results showed that the microbial alpha diversity based on Shannon index differed significantly between vineyards while it did not differ between two grape cultivars. In both vineyards, *Proteobacteria* (27.76%), *Bacteroidetes* (13.37%)*, Actinobacteria* (8.16%) were the most represented bacterial taxa whereas the most represented fungal taxa were *Ascomycota* (30.8%), *Zygomycota* (16.25%) and *Basidiomycota* (8.61%). While bioavailable soil Cu concentrations varied amply between soil samples (from 2.77 to 50.7 mg kg^{-1}) we did not detect any correlation between Cu levels and rhizospheric microbiome diversities. Our results contribute to understand the complexity of the soil-microbiome interactions in the perspective of understanding their impact on soil fertility, plant physiology and crop systems sustainability.

In the second study, we were focusing on different wheat species and genotypes such as Bread Wheat, Wild Emmer Wheat, Domesticated Emmer Wheat, Durum Wheat Landraces, Durum Wheat cultivars, *T. monococcum* and triticale in two fields located in Bologna and Foggia. Fields presented different managements, in which the field of Bologna was under minimum tillage management and in rotation with *Fabaceae* family crops and the field of Foggia was under a conventional management with no rotation. The objectives of this research experiment were to elucidate and compare the rhizospheric and endophytic microbiota of 30 diverse wheat genotypes in two different fields using 16S amplicon sequencing, evaluate location and varietal effects, field management, plant developmental stage and other environmental factors shaping the microbiome communities. Our results showed that the microbial alpha diversity based on Shannon index differed significantly between fields of Bologna and Foggia, in which Bologna had a higher diversity in respect to Foggia for both rhizospheric and endophytic communities. The alpha diversity for rhizospheric and endophytic samples between majority of the wheat species within each field did not change significantly. However, there were some significant differences, for instance, in the rhizospheric samples between Bread Wheat and Wild Emmer Wheat in

Bologna. Thus, there was no notable change between wheat species of rhizospheric samples in Foggia. Furthermore, we have seen these microbiome differences at the level of plant species in endophytic samples in Bologna and Foggia. Using Shannon index there was significant differences, for instance, between Durum Emmer Wheat and Wild Emmer Wheat in Bologna, and between Bread Wheat and Durum Wheat Landraces in Foggia. We have also seen significant changes of microbiome diversity between different wheat genotypes in rhizospheric samples in Bologna and Foggia. In rhizospheric samples in Bologna, the most abundant phyla were *Actinobacteria* (28.02%), *Proteobacteria* (19.59%) and *Planctomycetes* (13.70%). As for Foggia, the first, second and third most frequent phyla were *Actinobacteria* (35.65%), *Proteobacteria* (21.61%) and *Chloroflexi* (12.02%). In endospheric samples the most frequent phyla in Bologna were *Proteobacteria* (38.06%), *Actinobacteria* (36.58%), and *Saccharibacteria* (8.31%). In Foggia we had the same three phyla as the most abundant taxa as following *Proteobacteria* (46.73%), *Actinobacteria* (38.76%), and *Saccharibacteria* (5.94%). Our results contribute to understand the role of wheat species and genotype and the filed management on the root-microbe-soil interactions in the perspective of understanding their impact on crop systems sustainability.

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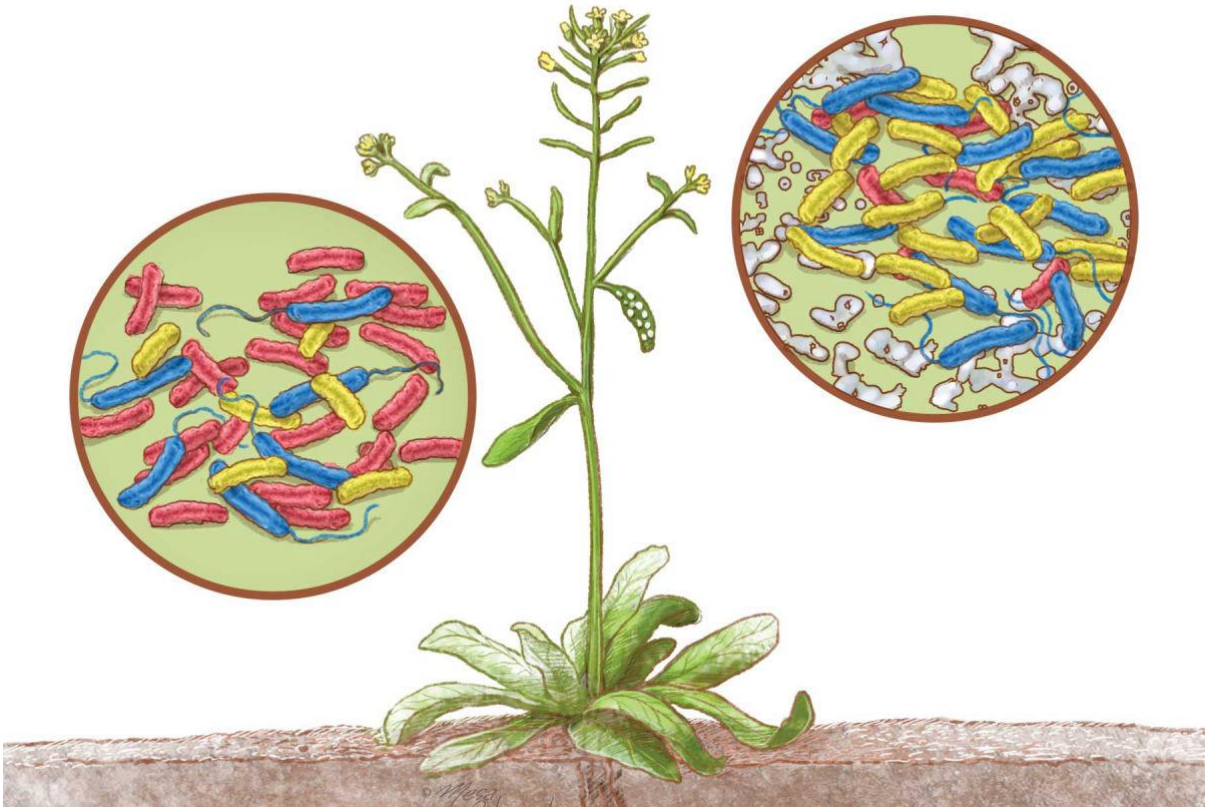
CHAPTER 1

INTRODUCTION: Plant Genetics – Microbiome Interactions



Chapter 1: Plant Genetics – Microbiome Interactions

1.1 Introduction

Soil biota possess a profound aptitude in altering nutrient cycling in their environment and accordingly supporting plant host for their primary productivity and its diversity (Haines-Young and Potschin 2010; Rousk and Bengtson 2014; Wagg et al. 2019; van der Heijden, Bardgett, and van Straalen 2008). The underground microorganisms including bacteria, fungi, protists, nematodes and viruses, interact with plants whether through the plant rhizosphere or root endosphere as plants grow, in varied ways such as via plant litter which it provides a nutritious habitat for plant growth and microbial diversity. Hence, there is strong evidence that multiple microbiome factors could affect both plant genotypes and phenotypes which these plant-microbiome interactions can be beneficial, neutral or phytopathogenic for the plant (Compant et al. 2011; Lau and Lennon 2012; Kardol et al. 2013; Terhorst, Lennon, and Lau 2014; Van Nuland et al. 2016). The plant-microbiome interaction can be seen as a reservoir of genes and essential functions for plant growth and fitness. Hence, this interaction is often positive for the plant and it promotes growth and protection from disease and stress, which is in the context of natural ecosystems and sustainable agriculture (Lemanceau et al. 2017). Learning about the importance of microbiome communities for the host plant, helps the management of these microbes for having an efficient and sustainable agroecosystem (Wallenstein 2017; Compant et al. 2019). The microbes are inhabitant in two plant regions known as rhizosphere and phyllosphere (Figure 1.1). Beside water, soil and air, the plant provides natural habitats for microbial growth. Rhizosphere is the soil region close to the plant roots which is influenced by plant exudates and oxygen availability. The rhizosphere differs from non-root-associated soil in its physical, chemical, and biological properties. Whilst, phyllosphere is all the aboveground plant organs, such as leaf, stem, flower and fruit. The microorganisms that are within the plant tissues, including roots, leaves and stem are known as endophytes. Depending on the plant compartment the microbiome diversity changes significantly, in which the region of soil that is in vicinity of plant root (i.e. rhizosphere) has higher diversity in comparison to the microbial communities within the plant organs (i.e. endosphere). In the same pattern, the microbes that colonize in aboveground plant organs (i.e. phyllosphere) has lower diversity in respect to rhizosphere microbes (Vorholt 2012; Bulgarelli et al. 2013; Trivedi et al. 2020).

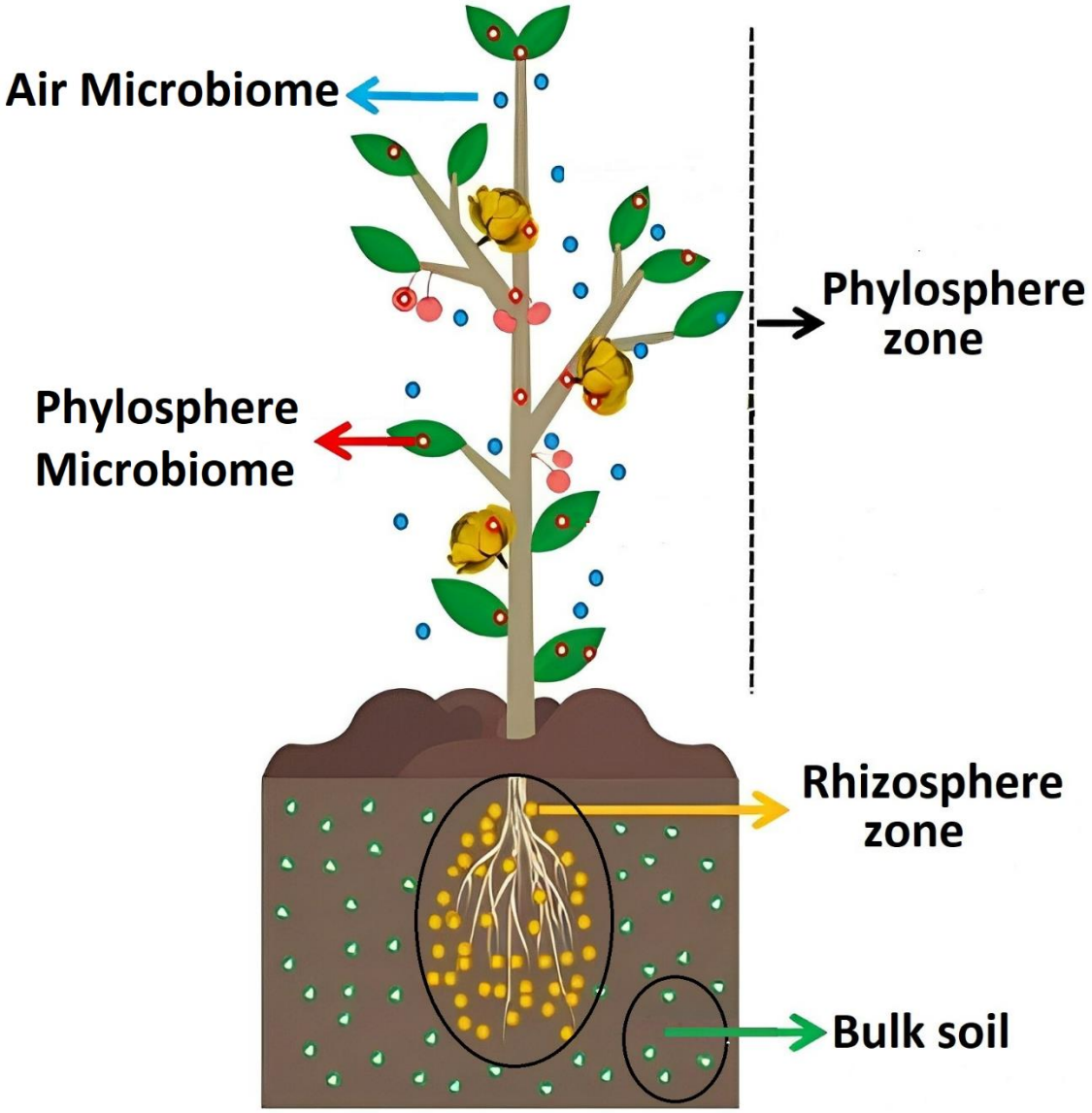


Figure 1.1 Pant Microbiome compartments.

Different studies are showing that the types of microbes are decreasing in epiphytic and endophytic microenvironments due to climate changes and agricultural practices because of their negative impact on soil biodiversity (Wagg et al. 2014; Bertola, Ferrarini, and Visioli 2021).

1.2 The Plant-Microbiome Interactions

Plants, like all living organisms, establish interactions with a multitude of microorganisms, in such a way that they affect both their development and their survival. Hence, all living organisms, including plants, can be considered as metaorganisms. Therefore, the plant depends on its microbiota to obtain certain benefits (obtaining nutrients and shielding against diseases) and in return, it provides them with nutrients in the form of exudates that can represent up to 21% of the

carbon that it fixes photosynthetically (Meharg 2012). Various studies consider these interactions crucial to understand the health of plants and their adaptation to both biotic and abiotic stress (J. Yang, Kloepper, and Ryu 2009; S. K. Upadhyay and Singh 2015), as well as a basic element to achieve sustainable crop protection (Berg et al. 2014).

The communication between the plant and the associated microorganisms, as well as the communication between the microorganisms themselves (Hosni et al. 2011), occurs through the perception of quorum (Quorum Sensing). It is a gene regulation mechanism involved in microbial activities such as antibiotic production, biofilm formation, conjugation, mobility, symbiosis and virulence, among others (Miller and Bassler 2001; Rutherford and Bassler 2012; Wu and Luo 2021). Communication between Domains is produced by autoinducing molecules, such as AHL (N-acyl homoserine lactone) in Gram-negative bacteria (Mathesius et al. 2003; Hartmann and Schikora 2012). Plants respond to autoinducer molecules and are even capable of mimicking them (Teplitski, Robinson, and Bauer 2000; A. Gupta et al. 2022).

Imbalances or dysbiosis in the microbiome community can be the triggers for diseases. This dysbiosis can be caused by changing the abundance of microorganisms already present or by other microbes transported by air or by animals. Plants can present resistance to these diseases, which is defined as the ability to suppress an infection, its development and/or the reproduction of parasites (Roberts 2002). Resistance can be due to the plant's own genes (Williamson and Roberts 2009) or induced by interactions with microorganism community (Schouten 2016). These microorganisms that help suppress a disease are called biological control agents or biocontrol agents (BCA). Biological control agents can use one or several mechanisms, such as antibiosis, mycoparasitism, competition and induction of generalized resistance in the plant (Elad and Freeman 2002; Shoresh, Harman, and Mastouri 2010).

1.3 Organic compounds (Exudates) released by the roots

The roots release considerable amounts of organic carbon into the rhizosphere. This carbon is acquired by the plant through the process of photosynthesis and a large proportion of this fixed carbon (between 30-60% in annual plants) is transported to the root system (Lynch and Whipps 1990). In the carbon fixation network, the percentage lost by root exudation is 17% (Nguyen 2003).

The exudation has important consequences for the activity and composition of microbial communities, the availability of nutrients and the solubility of toxic compounds in the rhizosphere (El-Shatnawi and Makhadmeh 2001; Sudhir K. Upadhyay et al. 2022). Many studies, mainly with annual plants grown under controlled conditions, have shown that various plant species differ in

the amount and composition of exudates (Petra Marschner, Grierson, and Rengel 2005; Herz et al. 2018; Dhungana, Kantar, and Nguyen 2023).

The chemical components released by the root can be classified into two groups depending on their mode of excretion:

(1) Water soluble exudates that are low molecular weight compounds such as: sugars, amino acids, organic acids, hormones, vitamins, carboxylates, siderophores, phenols, and gases such as ethylene, CO_2, and HCN are released down a concentration gradient.

(2) High molecular weight substances such as polymeric carbohydrates and enzymes that depend on certain metabolic processes for their release, lysates that are released when cells autolyse, including the cell walls and eventually the whole root and mucilage that is made up of sugars (arabinose, galactose, fructose, glucose and xylose) and polygalacturonic acid in addition to 6% of proteins which are secreted from the root sheath as a gelatin whose function is to protect the root meristem and improve soil contact with the root. The mucilage with inclusions of soil particles and microorganisms is what is known as mucigel. The production of these compounds is positively related to root growth (El-Shatnawi and Makhadmeh 2001; Neumann and Römheld 2002; Nguyen 2003; Bais et al. 2006).

1.4 Bacterial communities in rhizosphere

A specific form of bacterial communities can be found growing in the rhizosphere and it is universally accepted that members of a specific group can perform important functions in the ecosystem (Barea et al. 2005). The quantitative and qualitative nature of rhizosphere-rhizoplane microorganisms is subject to many factors that influence their structure and species composition; therefore, it should not surprise us that microbiome communities are modified by many factors that also affect root exudation such as soil type, plant species, nutritional status, age, stress and other environmental factors (C. H. Yang and Crowley 2000; Duineveld et al. 2001; Kawasaki et al. 2016; Ling, Ma, and Zhang 2022).

Plant development has a great influence on the structure of microbial composition in the rhizosphere (Lundberg et al. 2012; Chaparro, Badri, and Vivanco 2014). It has been found that the stage of plant development alters the bacterial community, and the state of maturity controls the magnitude of the rhizosphere effect and the degree of response by specific microorganisms (Morgan, Bending, and White 2005). This type of alterations has been recorded in very young seedlings, allowing us to observe that organisms that present rapid growth rates (r - selection) respond to root excretions during this stage of development. While during the late development, decomposing tissues contribute appreciably to the dominance of slow-growing bacterial communities (K - selection) capable of degrading more complex substrates.

Moreover, soil physics also have strong effects on the microbial composition. The texture of soil could limit the availability of root exudates in the soil, and consequently affecting the survival and growth of microorganisms (Garbeva, van Veen, and van Elsas 2004).
In the same way, cultivation practices such as rotation and tillage generate variation in microbiome communities (C. H. Yang and Crowley 2000; Navarro-Noya et al. 2013; Delitte et al. 2021; Z. Yuan et al. 2022).
The growth and metabolic activity of the microbial composition in the rhizosphere is affected by the availability of nutrients (Çakmakçi et al. 2006). Micronutrient factors such as nitrogen or iron can also affect the number of bacteria and change their composition (P. Marschner et al. 2001; Carson et al. 2009; Finley et al. 2022).

The structure of the bacterial composition in the rhizosphere is important for plant activity. The interaction between bacteria and roots can be beneficial (growth stimulation), neutral, or harmful (pathogenesis, competition, and parasitism) (de Ridder-Duine et al. 2005; Bais et al. 2006). Such interactions can influence plant growth and development, change nutrient dynamics, alter plant susceptibility to disease and abiotic stress processes (Morgan, Bending, and White 2005). Microorganisms in the rhizosphere exert strong effects on plant growth and health, either through the formation of growth-stimulating substances (biofertilizers), nutrient solubilization, nitrogen fixation, and the production of phytohormones (Grayston et al. 1998; Barea et al. 2005), and moreover, the production of antibiotics that results in the suppression of pathogenic microorganisms by antagonistic bacteria (Garbeva, van Veen, and van Elsas 2004) and the bioremediation of environments (Kapley, Prasad, and Purohit 2007; Desai, Pathak, and Madamwar 2010; L. M. Coelho et al. 2015).

Bacteria that provide some benefits to plants are divided into two different types: those that establish a symbiosis with the plant (Rhizobia) and those that live freely in the soil but are commonly found near or even within the roots due to their energy dependency, which are called Plant Growth Promoting Rhizobacteria (PGPR) (Glick 1995; Bais et al. 2006). Therefore, microbial metabolites interfere with plant metabolism, and whether the produced substances are beneficial or toxic can cause immediate physiological responses of considerable magnitude (Ortíz-Castro et al. 2009; Canellas and Olivares 2014). Some bacterial taxa considered as PGPR includes species of *Azotobacter*, *Azospirillum*, *Actinobacteria*, *Bacillus*, *Pseudomonas*, *Acetobacter*, *Lactobacillus* and *Burkholderia* (Babalola 2010; Santoyo, Orozco-Mosqueda, and Govindappa 2012; Lamont et al. 2017; Shivlata and Satyanarayana 2017).

Finally, understanding how all these processes in a microbiome community affect the ecosystem is a vital matter in microbial ecology (Miethling et al. 2000). In addition, understanding the structure

of plant-associated bacterial compositions and their alteration over time is essential for a better realization of mechanisms concerning the plant growth-promoting microorganisms in the rhizosphere (Di Battista-Leboeuf et al. 2003).

1.5 The roles of Plant Growth-Promoting Rhizobacteria (PGPR) in plants

The soil is a natural ecosystem in which numerous different microorganisms proliferate (Cassán et al. 2009). The possibility of using soil and root microorganisms that favor plant nutrition and development offers new alternatives to increase yield and improve the use of mineral fertilizers (Fuentes-Ramirez and Caballero-Mellado 2006). As an alternative solution to the problems of nutrient depletion in the soil and its contamination due to excessive use of fertilizers and the need to meet the demand for chemical fertilizers for food production, the FAO, within the first principle of "Building a common vision for food and sustainable agriculture", as an alternative proposed the "use of microorganisms in the cycling of nutrients" (Food and Agriculture Organization 2014). The bacteria that inhabit rhizosphere or the soil zone that is influenced by the roots are called Rhizobacteria (Cassán et al. 2009). The interactions and communication between root and microorganisms in rhizospheric zone play a very important role in maintaining plant growth and productivity (Hayat et al. 2010). These microorganisms are capable of increasing the absorbance and/or transforming not accessible elements in suitable forms for plant cell. Kloepper and Schroth in 1978 introduced the term "Plant growth promoting rhizobacteria (PGPR)" for these beneficial microorganisms which led the way for further discoveries.

PGPRs are bacteria isolated mainly from the rhizosphere (Bashan et al. 2014) and promote plant growth through a wide variety of mechanisms (Pii et al. 2015). Several studies highlighted that the inoculation of plants with PGPR can have considerable effects on the plant at both physiological and molecular levels, suggesting the possibility that soil biota may stimulate plants to be more efficient in the recovery of soil nutrients (Pii et al. 2015; Khan et al. 2019; Khan, Bano, and Babar 2020).

Moreover, essential macronutrients such as Nitrogen (N), phosphorus (P) and potassium (K) are not always available to plants in the soil. The PGPRs improve the availability of these nutrients through the mechanisms of biological nitrogen fixation, reduction of N_2 to NH_3 by the nitrogenase enzyme complex, and the solubilization of P and K through the release of organic and inorganic acids. PGPR can be classified as biofertilizers when they act as a source of plant nutrition and a source of enrichment to replenish or rebuild the nutrient cycle between the soil, plant roots and other microorganisms. PGPRs increase the availability of nutrients in the rhizosphere by influencing plant metabolism and improving their nutrition through direct mechanisms such as:

synthesis of phytohormones (auxins, gibberellins, cytokinins), vitamins and enzymes; sulfide oxidation; increase in root permeability; nitrite production; accumulation of nitrates; reduction of heavy metal toxicity and 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme activity and solubilization of the non-accessible K form from minerals (Dobbelaere, Vanderleyden, and Okon 2003; X. Hu, Chen, and Guo 2006; C. Zhang and Kong 2014; Han et al. 2015; Meena et al. 2015; Setiawati and Mutmainnah 2016; Ghorai et al. 2021).

1.6 The genomic basis of plant-microbiome

Deciphering the prevailing molecular mechanisms of plant-microbiome interactions is crucial to understanding the beneficial or pathogenic effects that the microbiome can exert on the plant. Studies based on "omics" tools and next-generation sequencing (NGS) technologies have proven to be valuable tools for studying microbiome-host interactions (Imam, Singh, and Shukla 2016). In recent years, it has become evident that the structure of the plant-associated microbiome plays a central role in plant health (Bulgarelli et al. 2013). Given their important role in sustainable agriculture, emphasis has been placed on the study of these microbiomes and their interactions with plants. In this sense, NGS technologies have provided new insights into the composition and organization of the plant-bacterial microbiome associated with *Arabidopsis*, *Populus* and *Zea mays* plants (Bulgarelli et al. 2012; Lundberg et al. 2012; Shakya et al. 2013; Peiffer et al. 2013). In particular, the detailed characterization of the endophytic microbiome associated with the *Arabidopsis* root, showing that the dominant phyla within it (i.e. endophytes) are less diverse than those associated with the rhizosphere soil around the root (Bulgarelli et al. 2012; Lundberg et al. 2012). Furthermore, it was observed that the endophytic microbiome representing a variation depending on the genotype within the *Arabidopsis* species, and a higher variation when other related species are evaluated (Guerrero, Margulis, and Berlanga 2013; Hardoim et al. 2015; Lemanceau et al. 2017). However, the importance of combining both crop-dependent and crop-independent methods to characterize the microbiota of plants has been reported (Anguita-Maeso et al. 2019), evidencing that no method can capture the plant microbiome in its entirety. This seems to be especially relevant for the characterization of the endophytic microbiome inhabiting in nutritionally poor environments, such as xylem vessels.

1.7 Metagenomic techniques for the study of microbial communities

There are numerous differences between the various organisms that make up a microbial community, and biotechnological advances have revealed that it is possible to use the metabolites of any of them for the production of antibiotics, PGPRs, vaccines and others. The cultivation, isolation and description of microorganisms has been essential to decipher the characteristics of

the different microbial species (Bilen et al. 2018). However, the use of new technologies has been needed to understand different factors in the behavior of microbial communities, since not all its members can be cultivated with currently known techniques. The use of culture-independent molecular tools, in combination with the application of ecological theory, has been key to advancing the understanding of the structure and function of complex microbial systems (Verstraete et al. 2012). The metagenomics can be given by two types of sequencing strategies: amplicon sequencing or massive random sequencing (shotgun). The first one uses a marker gene, typically the one that codes for the 16S subunit of ribosomal ribonucleic acid (RNA). This gene has made a significant progress in microbial studies due to the possibility of extensive characterization of the microbial communities' structure in high diversity ecosystems. This achievement has been done mostly through the sequencing of amplicons from one or more of the 9 hypervariable regions (V1-V9) of 16S rRNA gene (Huse et al. 2008).

Shotgun sequencing allows to study the functional role of the presented taxa in the community, in addition to their genomic characteristics. In this way, it is possible to establish functional relationships between the different microbial members and understand both their presence and the metabolic interactions that occur.

1.8 Marker genes and metagenomics

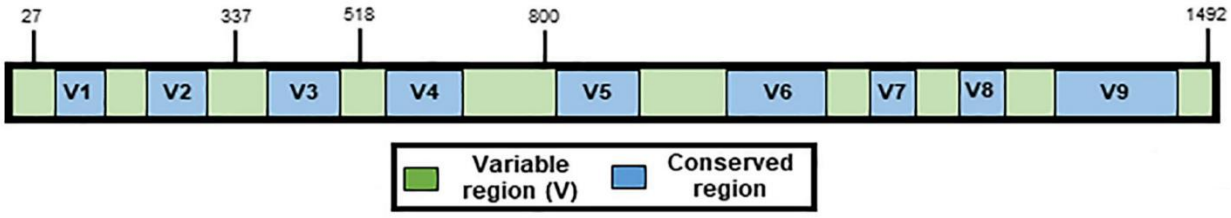
Metagenomics is responsible for studying the set of genomes of a community from a total DNA extraction, and it is culture-independent. The first metagenomic studies were based on Sanger sequencing. The first step was the segmentation of the genomic DNA into multiple random fragments. These fragments were then cloned into large plasmids such as fosmids, cosmids, and artificial chromosomes (BACs) that grow in monoclonal libraries to obtain enough genetic material for sequencing. From there, the DNA was sequenced (fluorophore sequencing coupled to the primers) (Slatko et al. 2011). Since its advent, Next Generation Sequencing (NGS) technologies have rapidly gained ground over Sanger sequencing. This type of sequencing allows obtaining millions of reads in a single run without the need to clone the fragments to be sequenced, therefore it optimizes the work in time and cost (Hall 2007). One of the major limitations of this technique is the size of the fragments, which are shorter compared to previous sequencing technology. However, its high sequencing depth represents a clear advantage. At the same time, it poses a technical challenge, since the processing and analyzing of millions of reads is not trivial. It is for this reason that there is an urgent need for constant bioinformatics development (Meyer et al. 2008; Huson et al. 2011; Gerlach and Stoye 2013). Particularly in environmental samples, random mass sequencing generates millions of reads from hundreds of different species,

presenting an even greater challenge. The use of marker genes to study the structure of different communities has been a great advance in the study of microorganisms. The 16S ribosomal gene (16S rRNA) and ITS have been the most widely used for the study of taxonomic comparisons in prokaryotes and eukaryotes, respectively. 16S ribosomal gene has an essential function in protein synthesis, and is present in all prokaryotic members. These approximately 1500 base pair (bp) sequences (Figure 1.2) are different between species, but are conserved within a single species which makes it suitable for assessing genetic diversity within microbial communities and for establishing phylogenetic relationships between different organisms. The conserved sequences have allowed the identification of universal primers that are used in DNA amplification reactions (Baker, Smith, and Cowan 2003). These characteristics letting to establish a precise taxonomic classification. The classical method for the study of microbial communities from marker genes is to use a variant portion thereof and amplify it by the polymerase chain reaction (PCR) technique. In this way, numerous copies of the same gene are obtained, thus facilitating its subsequent sequencing. In the last decade, the sequencing of the entire metagenome in short reads allowed us to analyze in more detail the different microbial communities. Moreover, in recent studies, information from reads of the entire genome is used to reconstruct marker genes, such as 16S rRNA, and thus study the composition and diversity of the community. Random massive sequencing (shotgun) does not go as deep as in the case of marker gene amplification; however, it has been shown to be representative of the community (Durazzi et al. 2021; Peterson et al. 2021). On the other hand, the use of shotgun sequencing does not have PCR errors that can result in biases in community analysis (Tremblay et al. 2015).

Figure 1.2 Schematic representation of the bacterial gene encoding ribosomal RNA (16S rRNA). Variable regions are highlighted in green, while conserved regions are highlighted in blue.

1.9 Conclusion

Due to importance of microorganisms for improving plant stress resilience, growth and health many microbial approaches have been developed, which some are outweigh the others. The new



generation of plant breeding approaches and the selection of proper agricultural practices that favoring the beneficial microbes is an essential approach for sustainable agriculture. In the last years, many studies increased our knowledge regarding plant microbiota and its functionality.

Hence, it is extremely important to constantly increase our understanding of the plant microbiome interactions in different crops and environments to improve the plant performance and its quality and applying effectively these processes in management and agricultural practices.

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CHAPTER 2

Absence of Correlation Between Bioavailable Copper Levels and Microbiome Diversity Based on 16S rRNA Genes and ITS Region in Grapevine Rhizosphere

Chapter 2: Absence of Correlation Between Bioavailable Copper levels and Microbiome Diversity Based on 16S rRNA Genes and ITS Region in Grapevine Rhizosphere

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Manuscript in prepration

2.1 Introduction

Soil has a significant role in shaping and sustaining biodiversity on earth, including providing a physical substrate for agriculture. Soil microorganisms are fundamental to soil sustainable functioning of natural and managed ecosystems, as they could affect productivity and biodiversity of aboveground ecological communities (Delgado-Baquerizo et al. 2016; Pantigoso, Newberger, and Vivanco 2022; French et al. 2021). The rhizosphere is a narrow zone of soil surrounding and influenced by the roots and heavily populated by microorganisms (Reinhold-Hurek et al. 2015). Many factors contribute to shape such communities including geographical locations, soil characteristics, crop managements and plant species (Bulgarelli et al. 2012; Peiffer et al. 2013; Coleman-Derr et al. 2016; Wang et al. 2017; Poudel et al. 2019; Longley et al. 2020; Burns et al. 2015). Within-plant species genetic variation was also found to have an effect on rhizospheric microbiome although in several studies this effect appeared to be smaller than the one generated by edaphic and environmental factors (Fierer 2017; Edwards et al. 2015; Walters et al. 2018; Bergelson et al. 2021).

Grape (*Vitis vinifera* L.) is one of the cultivated crops with the greatest economic importance (Alston and Sambucci 2019), however we still have limited information on the role of root microbiome on water and nutrients uptake, disease pressure, and other physiological traits up to grape production (Zarraonaindia et al. 2015). The type of rootstock was generally shown to affect bacterial and fungal communities, substantiating the role of host plant genetics in shaping the grape root microbiome (Wright et al. 2022; Marasco et al. 2018; Berlanas et al. 2019), although in at least one study the rootstock effect was not detected (Cureau et al. 2021). The effect of geography, local spatial and temporal (eg. vineyard age) dynamics were also investigated and shown to have effects on microbial community although some time with contrasting results (Manici et al. 2017; Berlanas et al. 2019; Martínez-Diz et al. 2019).

Copper (Cu) is an essential element for plants, with roles on photosynthetic and respiratory electron transport and is at the same time potentially phytotoxic (Kumar et al. 2021; Mir, Pichtel, and Hayat 2021). Cu is also used for control of fungal and bacterial diseases such as downy

mildew in grape, potato late blight, apple scab and others in conventional and organic farming, hence, its environmental concentration is increasing, including in vineyard soils (Brun et al. 2001; Pietrzak and McPhail 2004; Roviello et al. 2021; Ballabio et al. 2018; Genova et al. 2022). Being a heavy metal, Cu is a non-degradable pollutant, therefore its correct management is crucial in the perspective of improving cropping systems sustainability (Briffa, Sinagra, and Blundell 2020). Likewise, high soil copper concentration was shown to affect the belowground prokaryotic and eukaryotic communities and to change their diversity (Nunes et al. 2016; Rocca et al. 2018; Fagnano et al. 2020). It should be finally noted that plants can modify their root exudation in order to change root-associated microbiome and protect from the adverse effects of soils contaminated by heavy metals (Huang et al. 2014).

Our study was aimed at implementing next-generation sequencing (NGS) of targeted amplicons protocol (Franzosa et al. 2015) to (1) unravel the grape rhizobiota diversity between two cultivars and locations and (2) assess the correlation between bioavailable Cu levels in soil and microbiota diversity in vineyards.

2.2 Materials and methods

2.2.1 Soil sampling and characterization

Samples were collected from two northern Italian commercial vineyards, namely Molinari and Picozzi. Molinari is located in Valsamoggia municipality (44°33'43''N; 11°07'23''E) and is conducted under integrated pest management method. In this farm, samples were collected from a field cultivated with two grapevine cultivars namely 'Alionza' and 'Pignoletto', grafted on local ancient different rootstocks. Picozzi is located in Ozzano dell'Emilia municipality (44°23'46''N; 11°26'02''E). In this farm, a vineyard planted with 'Pignoletto' grafted on SO4 rootstock, and cultivated according to the integrated pest management methods was targeted for sampling. Rhizospheric soil samples were collected in February 2018 from both vineyards, from a 10-cm soil depth from the soil surface according to a nonsystematic W-like pattern sampling and from both sides of the grapevine rows. In the Molinari vineyard, 28 soil coring samples were collected for both Pignoletto and Alionza cultivars, from seven randomly selected plants per cultivar and from two different locations of the field (eastern and western side) per plant. In the Picozzi vineyard, 20 soil coring samples were collected from 10 randomly selected plants of Pignoletto cultivar at the southern and northern side of the field.

2.2.2 Analysis of soil properties and elemental characterization

All soil samples were air-dried, grinded and sieved at 2 mm. Sieved samples were analyzed for pH (ISO 10390), electrical conductivity – EC (ISO 11265), total organic carbon – TOC (ISO

14235), total nitrogen - TN (ISO 11261), and total carbonates (ISO 10693). The soil samples were analyzed for pseudo-total and potentially available-to-plant (bioavailable) metal content as well as metagenomics analysis. Pseudo-total concentration of trace metals was assessed on air-dried samples by nitric acid/chloric acid (1/3 v/v) digestion and analyzed by ICP-OES (ISO 12914, ISO 22036). Bioavailable content of trace metals was assessed on air-dried samples by diethylenetriaminepentaacetic acid (DTPA) extraction at pH 7.3 and analyzed by ICP-OES (ISO 17402:2008).

2.2.3 Genomic DNA extraction, 16S rRNA Gene and ITS region Amplicon Library Preparation from grapevine rhizosphere soil

The extraction of genomic DNA from soil samples was carried out using QIAGEN DNeasy PowerSoil kit (Qiagen, Hilden, Germany). Following standard quali-quantitative checks, genomic DNA samples were subjected to PCR for taxonomic informative gene loci (16S locus for archaea and bacteria and ITS region for fungi). Fragment of the bacterial 16S rRNA gene (V3-V4 region) were amplified with the following primer set: 341F: 5'-CCTACGGGNGGCWGCAG-3' and 805R: 5'-GACTACHVGGGTATCTAATCC-3'. The fungal ITS fragments (ITS1 and ITS4 regions) were amplified using the following primer set: ITS1 5'- TCCGTAGGTGAACCTGCGG-3' and ITS4 5'-TCCTCCGCTTATTGATATGC-3' (White et al. 1990). PCR reactions were conducted in triplicates to reduce random effects, with 2.5 µl of template DNA in each reaction. 2x KAPA HiFi HotStart ReadyMix, along with the two indexed primers in a unique combination for each sample. PCR conditions were adjusted according to the guidelines; samples were initially denaturated at 95 °C for 3 minutes, then amplified by using 25 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 30 seconds. A final extension (72 °C) of 5 minutes ensured complete amplification. For the PCR clean-up 20 µl AMPure XP beads, 52.5 µl of 10 mM Tris pH 8.5, 400 µl of 80% Ethanol (per sample) have been used to purify the amplicons away from free primers and primer dimer species. Dual indices and Illumina sequencing adapters attached using the Nextera XT index kit. The second PCR reaction was performed using 5 µl of clean PCR amplicons as a template with 5 µl of Nextera XT Index Primer 1, 5 µl of Nextera XT Index Primer 2, 25 µl of 2x KAPA HiFi HotStart ReadyMix and 10 µl of PCR Grade water for 8 cycles at annealing temperature of 55 °C. After this step, the second PCR clean-up was performed. Then, the amplicons checked using a 2100 Bioanalyzer (Agilent, Santa Clara, CA).

After PCR amplification and NGS library preparation, sequencing of the samples was carried out on a MiSeq instrument (Illumina, San Diego, CA) using 300-bp paired-end reads. Raw reads were analyzed using QIIME2 Quantitative Insights into Microbial Ecology (v.2020.8.0), available publicly at http://qiime2.org/ (Bolyen et al. 2019). Quality control and denoising the data were

carried out using DADA2 package in QIIME2 (Bokulich et al. 2013; Pauvert et al. 2019; Kuczynski et al. 2011).

2.2.4 Processing of DNA sequence data

All raw FASQ files for 16S rRNA and ITS samples were analyzed and visualized using QIIME 2 (v.2020.8.0) (Bolyen et al. 2019). After importing the data using a Fastq manifest format for paired-end reads, the quality control and denoising were performed using DADA2 package for both 16S and ITS to trim and truncate the low-quality reads and filter out noisy sequences (Callahan et al. 2016). Furthermore, by using DADA2 algorithm the chimeric sequences and singletons were removed and sequences with$\geq 97\%$ similarity were assigned to the same ASV. Then, the phylogenetic tree was constructed in order to do the diversity analyses. To do that, a multiple sequence alignment using MAFT was performed and then the alignment filtered out using QIIME2 alignment plugin (Katoh and Standley 2013; Callahan et al. 2016). To annotate the taxonomic information of 16S rRNA and ITS region, SILVA 132 (https://www.arb-silva.de/) and UNITE version 8.0 (Kõljalg et al. 2013) databases were used for each representative sequence, respectively. The taxonomic classification was implemented after training the scikit-learn naïve Bayes machine learning classifier for classification of bacterial 16S rRNA and fungal ITS marker-gene amplicon sequences (Bokulich et al. 2018).

Diversity analyses (alpha and beta) were performed using QIIME2 plugins. For alpha diversity, the Shannon index was used to estimate both abundance and evenness of the taxa present for bacterial and fungal communities (Callahan et al. 2016; Anderson 2008), the Faith's PD index was used to measure the biodiversity that incorporates phylogenetic differences between species (Faith 1992, 2018), and also the Evenness index was applied to measure of relative evenness of species richness (Jost 2010; Pielou 1966). while the between samples beta diversity was evaluated by computing Bray-Curtis dissimilarity index and matrix, for both 16S and ITS datasets (Ricotta and Podani 2017; Pielou 1966).

To test the effect of the different factors (vineyard and cultivar) on bacterial and fungal communities composition, the Bray-Curtis dissimilarity values were subjected to an Adonis Test (PERMANOVA) (Anderson 2008) in the vegan package in R (version 4.0.3). Principal Coordinate Analysis (PCA) of Bray-Curtis dissimilarity matrix was also carried out in R. Moreover, correlation analysis was investigated between bioavailable Cu, total Cu, Cd, Co, Cr, Fe, Mn, Ni, Pb, Zn, pH and EC with microbiota diversities based on Shannon index for both 16S and ITS, by the non-parametric Spearman correlation in QIIME2 plugin (Y. Zhang et al. 2017).

2.3 Results

2.3.1 Soil analysis

A total of 48 soil samples were collected and analyzed for chemical-physical properties (Table 2.1). The range of variation of soil pH $(7.3 - 8.2)$, electrical conductivity $(EC: 0.22 - 0.25\ mScm^{-1})$, TOC $(11.52 - 18.90\ mgkg^{-1})$, TN content $(1.47 - 2.36\ mgkg^{-1})$ and total carbonates $(8.04 - 16\ \%\ dm)$ were typical of the geographical area under investigation (https://ambiente.regione.emilia-romagna.it/en/). In Molinari vineyard, significant differences for EC, Fe, Mn, Pb and Zn parameters were identified between Pignoletto and Alionza cultivars (Table 1). Moreover, other than Cr and Pb, most of the soil parameters between the two vineyards differed significantly (Table 1). As far as Cu was concerned, potentially bioavailable-to-plant Cu levels of soil samples from Molinari and Picozzi vineyards resulted significantly different (46.6 vs 2.77 mg kg^{-1}, respectively. $P < 0.05,$ Table 1). However, there was no significant difference between bioavailable Cu level between Pignoletto and Alionza cultivars in Molinari vineyard (42.5 vs 50.7 $mgkg^{-1}$ respectively. Table 2.1). These Cu values for both vineyards were within the range of values typically observed in Italian soil (https://ambiente.regione.emilia-romagna.it/en/; (Ballabio et al. 2018)).

Table 2.1 Results of chemical-physical analysis of soil samples from Molinari and Picozzi farms.

Parameter (unit)	Molinari 'Pignoletto'	Molinari 'Alionza'	Pignoletto vs. Alionza (t-test)	Picozzi 'Pignoletto'	Molinari vs. Picozzi (t-test)
$pH\text{-}H_2O$	7.9 ± 0.0	7.3 ± 0.3	0.01 $^{n.s}$	8.2 ± 0.3	113.5 ***
$pH\text{-}C_aCl_2$	7.5 ± 0.1	7.3 ± 0.1	-	7.6 ± 0.1	-
EC-($mScm^{-1}$)	0.23 ± 0.05	0.25 ± 0.03	11.1 **	0.22 ± 0.02	26.04 ***
TOC ($mgkg^{-1}$)	11.52 ± 0.74	18.90 ± 1.60	-	15.35 ± 0.12	-
TN ($mgkg^{-1}$)	1.47 ± 0.06	2.36 ± 0.22	-	1.76 ± 0.41	-
Total carbonates (% dm)	8.04 ± 0.30	11.49 ± 0.05	-	16.00 ± 0.93	-
Total Cu (mg/kg)	149.590	175.073	-	31.863	-
Bioavailable Cu (mg/kg)	42.521	50.730	3.14 $^{n.s}$	2.77	265.3 ***
Cd (mg/kg)	0.045	0.044	0.05 $^{n.s}$	0.017	99.6 ***
Co (mg/kg)	0.107	0.098	0.62 $^{n.s}$	0.0703	18.11 ***
Cr (mg/kg)	0.018	0.011	0.19 $^{n.s}$	0.006	1.29 $^{n.s}$
Fe (mg/kg)	9.267	24.23	20.1 ***	6.88	8.01 **
Mn (mg/kg)	26.354	21.57	5.81 *	9.273	160 ***
Ni (mg/kg)	0.830	0.888	0.49 $^{n.s}$	0.617	19.93 ***
Pb (mg/kg)	0.870	1.848	49.27 ***	1.022	0.93 $^{n.s}$
Zn (mg/kg)	4.196	18.796	20.25 ***	0.598	11.63 **
Textural class	silty clay loam	silty clay loam	-	silty clay	-

$^{n.s}$ = not significant; * $p - value < 0.05$; ** $p - value < 0.01$; *** $p - value < 0.001$; '-' = data was not available

2.3.2 Rhizosphere microbial diversity in grapevine

The same 48 soil samples were analyzed to evaluate the bacterial and fungal communities by 16S rRNA and ITS short-reads-based amplicon sequencing. An average of approximately 77,000 and 202,000 fragments (forward and reverse reads) for each sample of 16S rRNA and ITS, were obtained, respectively. After quality filtering and deletion of chimeric and singletons, rarefaction plots showed that stable results for both 16S and ITS were reached when approximately $> 1{,}000$ reads per sample were considered (Figure 2.1a-b). These results supported the robustness of our datasets.

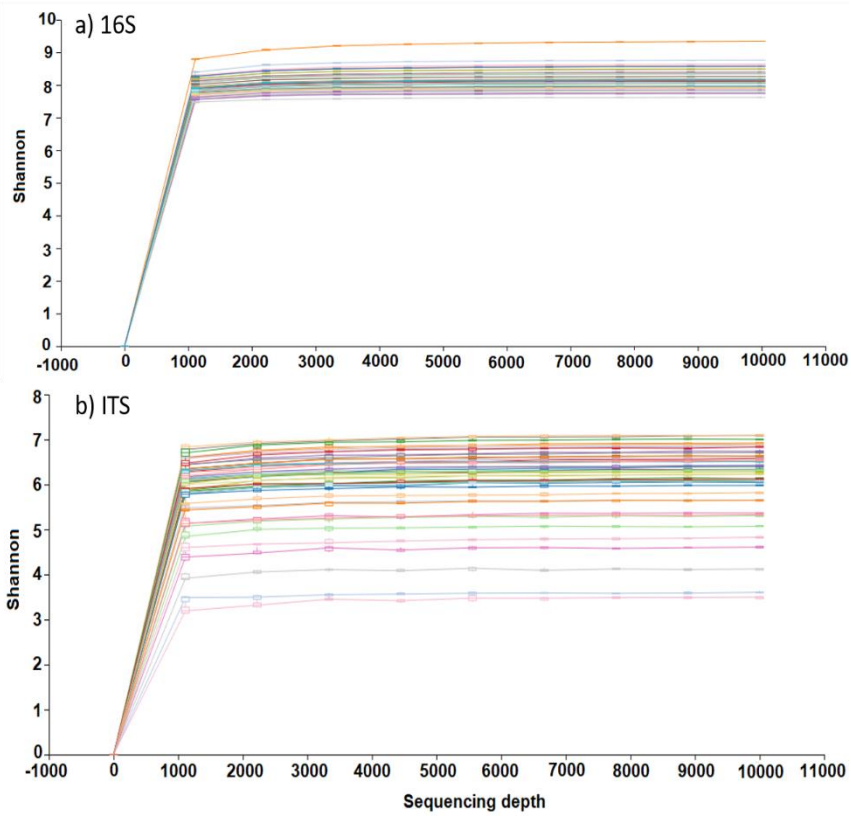

Figure 2.1 Alpha rarefaction plots based on Shannon diversity index for 16S (a) and ITS microbiome samples (b) in this study. A total of 48 samples were analyzed in both (a) and (b)

Alpha diversities of the soil samples from the Molinari and Picozzi vineyards and the two cultivars in Molinari vineyard were assessed using Shannon (H) index (Figure 2.2). The microbial diversity did not differ between vineyards for both bacteria and fungi (Kruskal-Wallis. Figure 2.2a-b). Within the Molinari vineyard, the two cultivars 'Alionza' and 'Pignoletto' did not show differences in both bacterial and fungal diversity (Kruskal-Wallis. Figure 2.2c-d). However, the overall Shannon index of bacterial communities was significantly higher than fungal communities (8.1 vs 6.3. Kruskal-Wallis, $P < 0.05$. Figure 2.2).

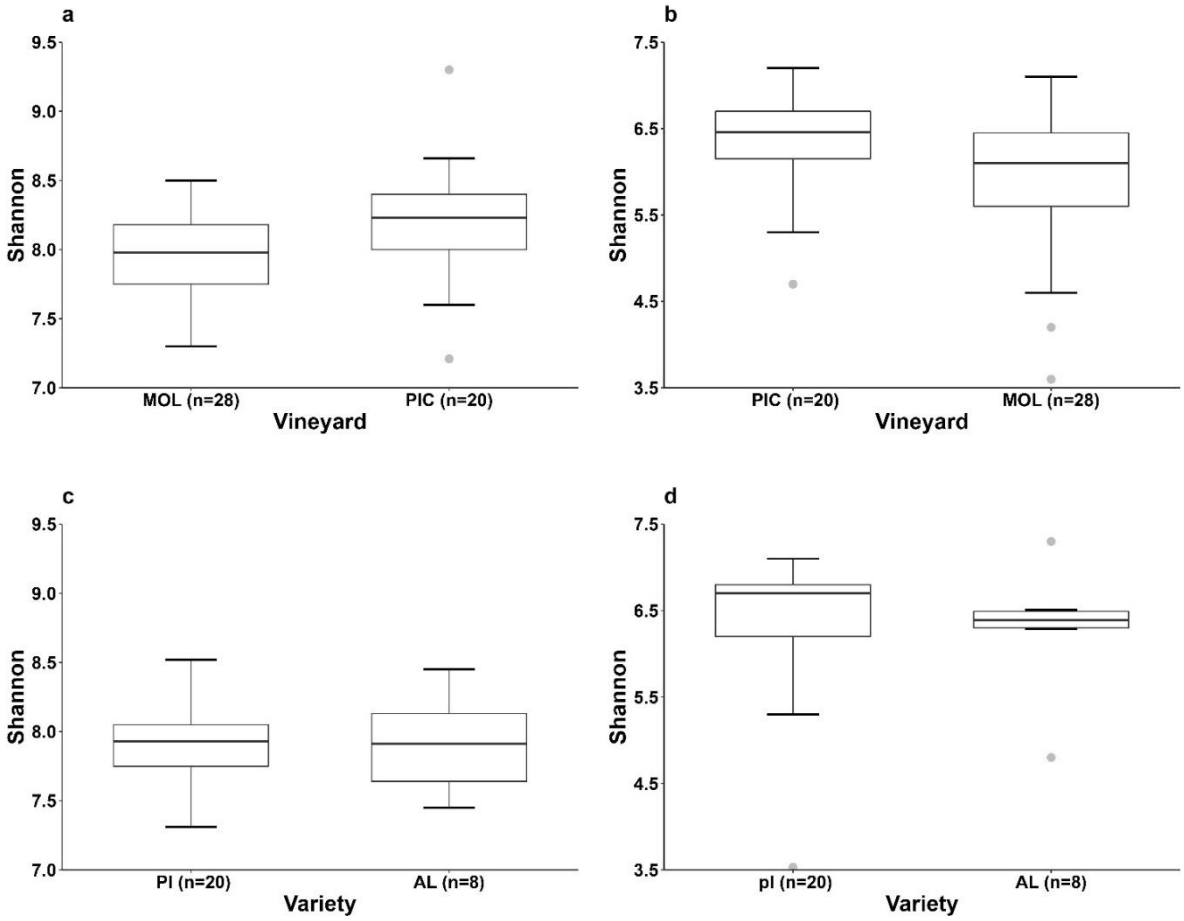

Figure 2.2 Alpha diversity (Shannon index) of bacterial and fungal soil communities. (a) 16S rRNA-based bacterial diversity estimated in the two vineyards, Molinari (MOL) and Picozzi (PIC); (b) ITS-based fungal diversity estimated in the two vineyards, Molinari (MOL) and Picozzi (PIC); (c) 16S rRNA-based bacterial diversity estimated in the Molinari (MOL) vineyard for two cultivars, Pignoletto (PI) and Alionza (AL); (d) ITS-based fungal diversity estimated in the Molinari (MOL) vineyard for two cultivars, Pignoletto (PI) and Alionza (AL).

To assess the microbiome diversities between vineyards and cultivars, Bray-Curtis dissimilarity index was computed for both 16S and ITS samples, and PCA plots were constructed based on the corresponding dissimilarity matrices (Figure 2.3). The PCA plots visualized a good division between Molinari and Picozzi soil samples for both 16S and ITS samples. An Adonis Test (PERMANOVA) showed that the vineyard was the most important factor in structuring bacterial (17%) and fungal (24%) communities. The Adonis result in Molinari vineyard showed that cultivar was the second most important factor in shaping bacterial ($R^2 = 0.09, P < 0.01$) and fungal ($R^2 = 0.11, P < 0.001$) communities (Table 2.2).

Table 2.2 Results of Adonis Test (PERMANOVA) for vineyard and cultivar factors

	Factor	Sum of Squares	R^2	P_value
Bacteria	Vineyard	1.04	0.17	0.001
	Cultivar	0.48	0.09	0.01
Fungi	Vineyard	2.82	0.24	0.001
	Cultivar	0.72	0.11	0.001

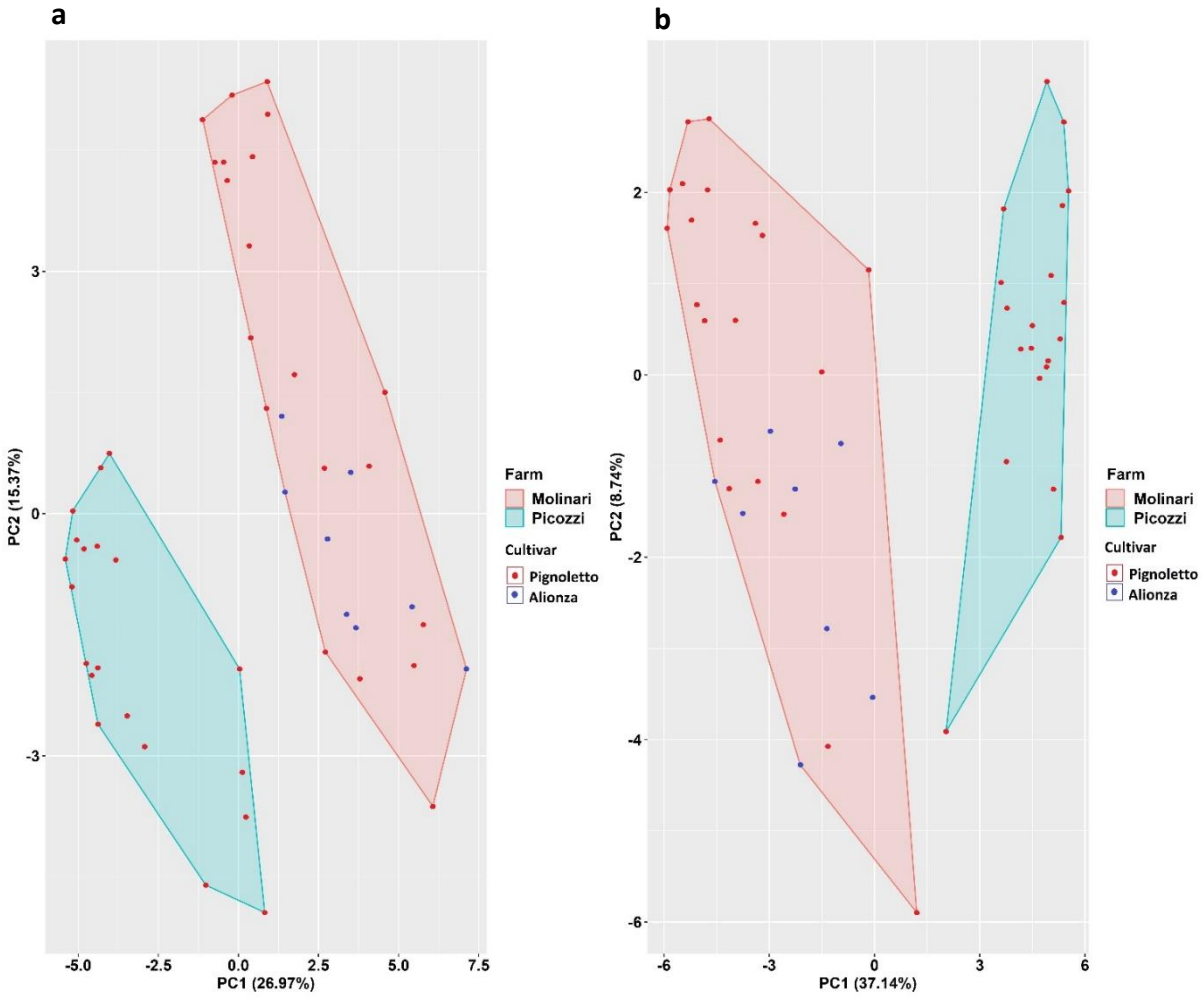

Figure 2.3 Principal component analysis (PCA) plots based on Bray-Curtis dissimilarity index for a) 16S and b) ITS. Molinari and Picozzi indicate the two different vineyards, 'Alionza' and 'Pignoletto' indicate the two different grape cultivars sampled within the Molinari vineyard.

2.3.3 Analysis of taxonomic distribution

The taxonomic distributions of rhizospheric bacterial and fungal communities for two vineyards and cultivars are given in Figure 2.4 at the phylum taxonomic level. Only groups with relative abundances $\geq 1.0\%$ were reported. As far as bacteria are concern, the first, second and third most frequent phylum in Molinari vineyard are *Proteobacteria* (28.45%), *Bacteroidetes* (13.71%) and *Actinobacteria* (7.98%), respectively. As for Picozzi vineyard, the first, second and third most frequent phylum are *Proteobacteria* (26.85%), *Bacteroidetes* (12.91%) and *Actinobacteria* (8.42%) (Figure 4a). In Molinari vineyard the first, second and third most frequent fungal phylum are *Ascomycota* (30.89%), *Zygomycota* (11.98%) and *Basidiomycota* (10.85%). Similarly, the first, second and third most frequent phylum in Picozzi vineyard are *Ascomycota* (30.68%), *Zygomycota* (22.21%) and *Basidiomycota* (5.47%) (Figure 2.4b). A large portion of bacterial (approx. 30%) and fungal (approx. 40%) sequences remained unassigned (Figure 2.4).

At the genus level, the most frequent bacterial genera across vineyards and cultivars were *Flavobacterium* and *Flavisolibacter* (*Bacteroidetes*), *Gemmatimonas* (*Gemmatimonadetes*), *Kaistobacter* (*Proteobacteria*), *Chthoniobacter* and *Pedosphaera* (Verrucomicrobia), *Gemmata* and *Pirellula* (*Planctomycetes*) (Table 2.3). The most frequent fungi genera were *Mortierella* (*Zygomycota*), *Tetracladium* and *Alternaria* (*Ascomycota*) and *Cryptococcus* (*Basidiomycota*) (Table 2.3).

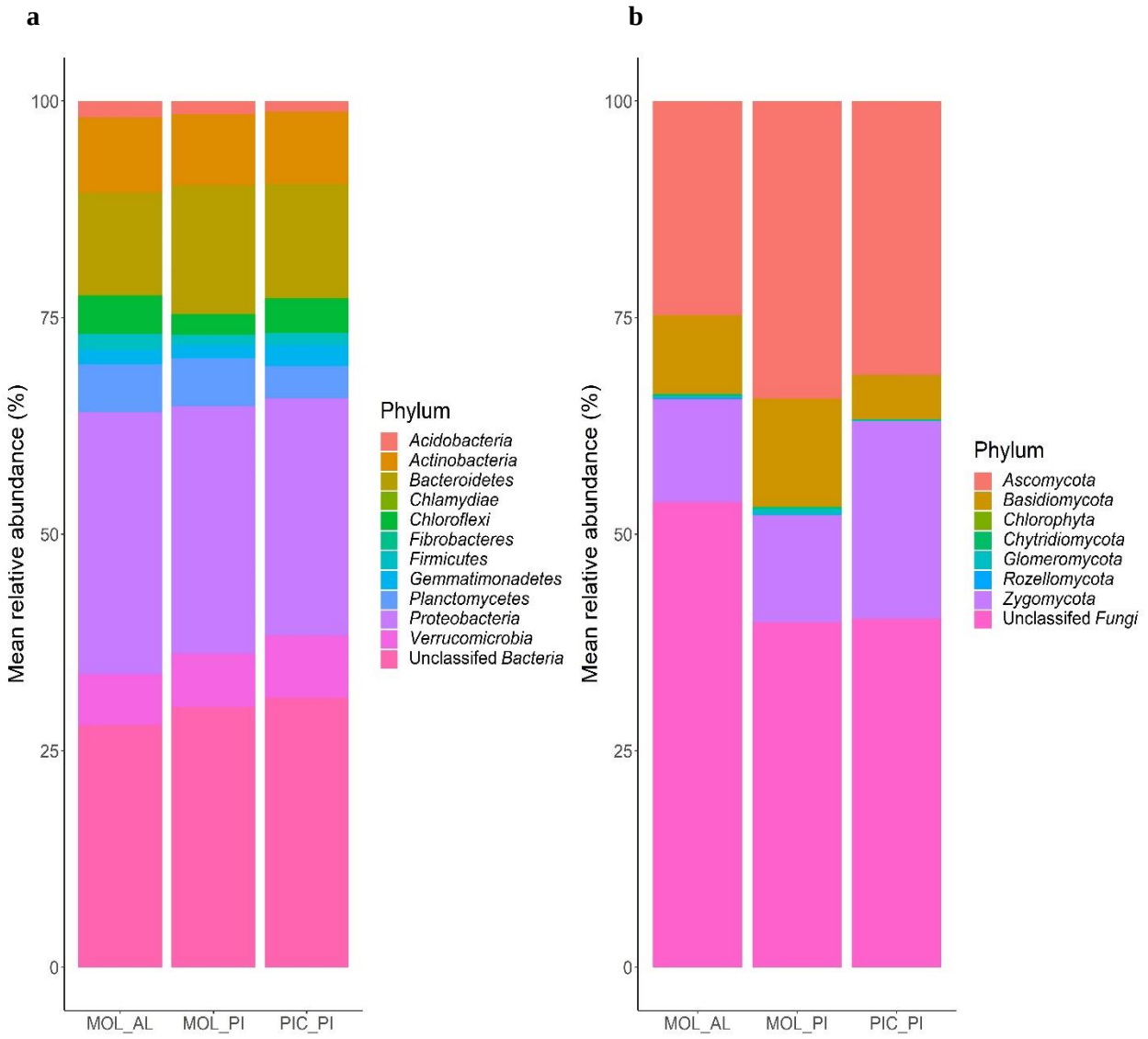

Figure 2.4 Taxonomy assignment bar plots at phylum level for (a) 16S, (b) ITS. On the X axis, MOL_AL = vineyard Molinari, cv. 'Alionza'; MOL_PI = vineyard, Molinari cv. 'Pignoletto'; PIC_PI = vineyard Picozzi, cv. 'Pignoletto'.

Table 2.3 Percentages of bacterial and fungal genera identified in the grapevine rhizosphere microbiota represented in more than 50% of the samples.

Type	Genus	Abundance % (*)		Representation % (**)	
		Molinari	Picozzi	Molinari	Picozzi
16S - Bacteria	*Flavisolibacter*	2.15	2.31	100	100
	Kaistobacter	2.01	2.37	100	100
	Flavobacterium	2.56	3.42	83	70
	Gemmatimonas	1.54	2.38	100	100
	Chthoniobacter	1.73	2.95	100	100
	Pedosphaera	1.52	1.79	100	100
	Gemmata	1.82	1.33	100	100
	Pirellula	1.79	1.25	100	100
ITS - Fungi	*Mortierella*	11.28	21.5	90	95
	Cryptococcus	7.82	1.56	93	100
	Tetracladium	2.82	3.68	83	100
	Alternaria	4.27	1.31	75	60

(*) The value is calculated as an average over all the samples in Molinari and Picozzi vineyards. (**) Percentage of genera where the abundance is ≥ 1.0%.

2.3.4 Evaluation of correlation between copper levels and composition of grapevine rhizospheric microbiota

Using QIIME 2 plugin, Spearman correlation was computed between soil parameters such as bioavailable Cu, Cd, Cr, Fe, Mn, Ni, Pb, Zn, pH and EC and microbiome diversity (Shannon, Evenness and Phylogenetic diversity Faith's PD) for Molinari and Picozzi samples separately (Table S1). The results showed that there was no correlation between bioavailable Cu and bacterial and fungal microbiota diversities in none of the two vineyards (Figure 2.5 and Table S2.1). Similarly, we did not observe any correlation between Cd, Cr, Fe, Mn, Ni, Pb, Zn, pH and EC and microbiome diversity using different indices (Table S2.1).

A relationship between soil copper levels and the abundance of specific taxa was also searched. Similarly, to previous results, no correlation was detected between *Proteobacteria* and *Ascomycota* phyla and bioavailable Cu concentration in Molinari and Picozzi vineyards (Figure 2.6). Further investigation on other 18 fungal and bacterial taxa in Molinari and Picozzi showed that there was no correlation between bioavailable Cu concentration and the fungal and bacterial taxa (Figures S2.1, S2.2, S2.3 and S2.4 on Supplementary materials).

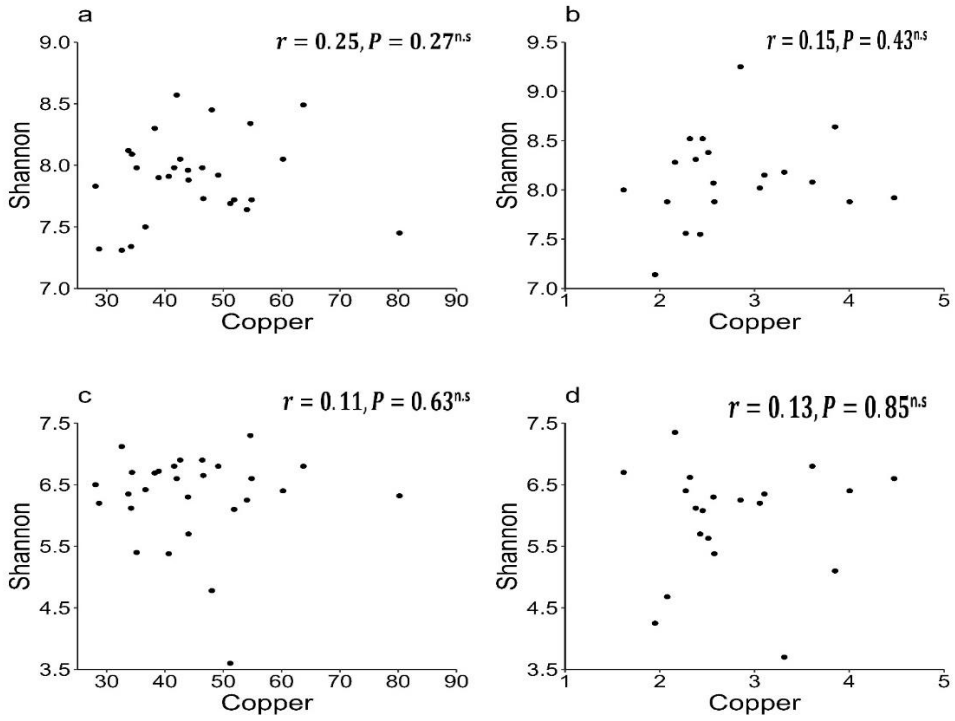

Figure 2.5 Scatter plots showing the correlation (Spearman) between the bioavailable Copper (express as mg kg^{-1} of soil) and microbiota diversities using Shannon index in: a) Molinari vineyard, 16S; b) Picozzi vineyard, 16S; c) Molinari vineyard, ITS; and d) Picozzi vineyard, ITS.

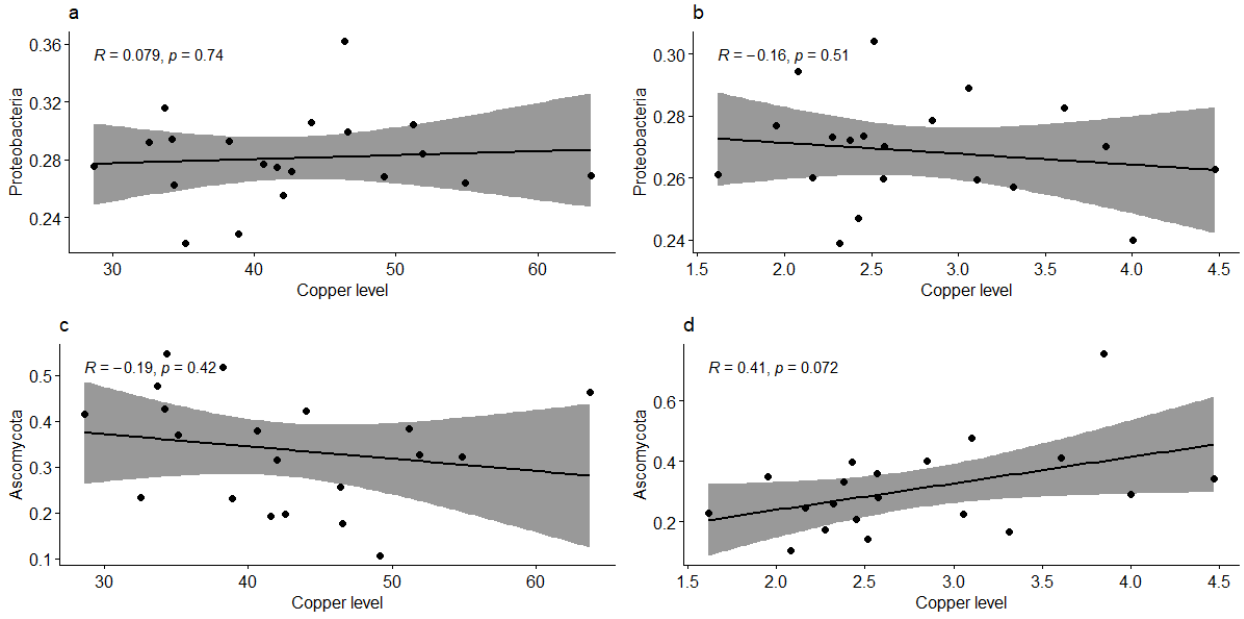

Figure 2.6 Correlation assessment of *Proteobacteria* and *Ascomycota* with bioavailable Cu in Molinari (a and c) and Picozzi (b and d) vineyards. In each plot, the horizontal and vertical axes show the copper level and relative frequency (RF) of the bacteria, respectively.

2.4 Discussion and conclusion

In this study we characterized the bacterial and fungal rhizosphere microbial communities in two vineyards and two cultivars and searched for correlations between microbiome communities and soil Cu levels. Soil chemical and structural analysis showed that the values and the variation ranges of soil pH, electrical conductivity (EC), TOC, TN content, total carbonates and bioavailable Cu were within the range of variation typical of vineyards and agricultural soils of Northern Italy (https://ambiente.regione.emilia-romagna.it/en/), thus our results can be considered of general interest. Additionally, the investigation of root microbiome composition and variation based on NGS target amplicon sequencing (16S rRNA V3-V4 hypervariable regions and ITS1 and ITS4 regions) appeared to be adequate as shown by the asymptotic plateauing of rarefaction curves. Among the PCR targets, we utilized 16S rRNA V4 region which was previously shown to be among the most informative target regions in metagenomic analysis (Youssef et al. 2009), thus providing support to our results.

In accordance with recent studies (Berlanas et al. 2019), our soil microbiome analysis revealed that the overall alpha diversity using Shannon index was higher in bacteria rather than fungi, and we have observed the same pattern in both Molinari and Picozzi vineyards with significant differences between the two vineyards. In a previous research study (Marasco et al. 2018) it has been showed that rootstock genotype could have a notable influence in shaping taxa in grapevine rhizosphere. This effect of host genotype shaping bacteria and fungi taxa has been reported in different woody crops such as, pines (Gallart et al. 2018), apple (Liu et al. 2018), as well as several annual crops, such as, potato (Inceoğlu et al. 2010), chickpea (Bazghaleh et al. 2015), and maize (Peiffer et al. 2013). This could be due to different genotype influences in root system, including exudate composition and immune responses, which may alter the microbiome compositions within the soil (Wagner et al. 2016). However, in our study, in Molinari vineyard we did not observe bacterial and fungal differences between the two varieties which could imply that the effect of genotype in shaping microbiome might be influenced by other factors. Furthermore, the beta diversity investigation showed that diversity from one vineyard to another one for bacterial and fungal communities was changing significantly. However, we did not find the same pattern between the two cultivars for 16S and ITS samples. These differences between Molinari and Picozzi vineyards could be due to several reasons including vary geographical locations and other environmental factors. In previous studies, soil physicochemical properties, organic matter and moisture content have been identified as the major influences of shaping the rhizosphere microbiome (Fernández-Calviño et al. 2009; Corneo et al. 2014; Burns et al. 2015; Zarraonaindia et al. 2015). Our taxonomical assignments showed that *Proteobacteria* and *Ascomycota* were dominant phyla in vineyard soil and had the highest relative frequencies in bacterial and fungal

communities, respectively and we have observed them through all the samples in both cultivars and vineyards. These results are in accordance with previous studies on grape (Vega-Avila et al. 2015; Zarraonaindia et al. 2015; Martínez-Diz et al. 2019; Berlanas et al. 2019; Wright et al. 2022). Besides, at genus level, the most frequent bacterial genera in two vineyards and cultivars were *Flavobacterium* and *Flavisolibacter (Bacteroidetes), Gemmatimonas* (*Gemmatimonadetes*), *Kaistobacter* (*Proteobacteria*), *Chthoniobacter* and *Pedosphaera* (*Verrucomicrobia*), *Gemmata* and *Pirellula (Planctomycetes*). The most frequent fungi genera in both Alionza and Pignoletto cultivars and the two vineyards were *Mortierella* (*Zygomycota*), *Tetracladium* and *Alternaria* (*Ascomycota*) and *Cryptococcus* (*Basidiomycota*).

The bioavailable Cu levels observed in this experiment in both Molinari and Picozzi vineyards were in range of 28.5 to 64 mg/Kg (equivalent to ppm) and 1.5 to 4.5 mg/Kg, respectively. Our results revealed a relatively strong difference in total and bioavailable Cu between the two vineyards, however the variability range are both observed in Italian and European vineyard soils which typically range from ca. 1 to a few hundred mg/Kg Cu (Ballabio et al. 2018).

Similar to a recent study (Fagnano et al. 2020), our result of non-parametric Spearman correlation test between bioavailable Cu levels and overall microbiota diversity using Shannon index showed that there was no significant relationship between the amount of bioavailable Cu and microbiome diversity neither for bacterial nor fungal communities in Molinari and Picozzi vineyards. Similarly, the further investigation for correlation between the most frequent bacterial and fungal taxa (*Proteobacteria* and *Ascomycota*, respectively) with the bioavailable Cu in both Molinari and Picozzi vineyards showed that there was no significant pattern. It should be noted that the two vineyards were in two rather different geographical locations. The two habitats used as vineyard sites were separated by approx. 45 km, and varied in most of physicochemical properties of soil. There is a universal regulatory pressure on agriculture in general, and specifically in organic farming systems to restrict the use of Cu (Wightwick et al. 2013). As previously found, high Cu concentrations could lead to environmental and human hazard (Pinto et al. 2014; Lamichhane et al. 2018; Steffan et al. 2018; F. C. Coelho et al. 2020; Guarino et al. 2020). Therefore, it would be wisely to limit the use of Cu in organic farming management due to its toxicity for soil and the potential risk pose to human's health. However, from a small-scale experiment, the results presented here, did not reveal any difference and benefit in excessive use of Cu for Pignoletto and Alionza grapevine cultivars and nor for the diversity of microorganisms in two vineyards. A better understanding of the vast diversity of bacteria and fungi in the interactions between microbiota and grapevine may facilitate the development of future strategies for grapevine production.

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CHAPTER 3

The Influence of Different Wheat Genotypes and Species on Rhizospheric and Endophytic Bacterial Communities

Chapter 3: The Influence of Different Wheat Genotypes and Species on Rhizospheric and Endophytic Bacterial Communities

3.1 Introduction

Cereals are annual cultivated grasses almost all belonging to the *Poaceae* family, which produce starchy, floury, edible seeds for numerous food preparations. Cereals are the most important group of crops in the world agricultural economy, and the diet of most civilizations has always been based on cereals cultivation (Dolezel et al. 2007; Awika 2011). From a nutritional point of view, although cereals have a highly variable protein content (8-15% of the dry weight of the kernel), they mostly lack essential amino acids such as lysine, threonine and tryptophan (Welch 2005; Deleu et al. 2019).

One of the most cultivated cereals in the world is wheat. Its wide adaptation to diverse environmental conditions is possible due to the great existing genetic variability, represented by more than 2500 cultivars (Shewry, Halford, and Lafiandra 2003; Mondini et al. 2010). Wheat identifies different species belonging to the genus *Triticum*, which are distinguished according to the number of chromosomes in:

- diploids with $2n = 2x = 14$. They contain the A genome (for instance, *T. monococcum*, *T. aegilopoides*, *T. beoticum, T. urartu*);
- tetraploids with $2n = 4x = 28$. They contain the genomes AB (for instance, *T. turgidum* ssp *durum, T. turgidum* ssp *dicoccum, T. turgidum* ssp *polonicum*) or AG (*T. timopheevii*);
- hexaploids with *2n=6x=42*. They contain the genomes ABD (*T. aestivum and T. aestivum* ssp *spelta*) or AAG (*T. zhukowskyi*).

In the evolutionary history of wheat, the following species are particularly relevant: *T. urartu* (Einkorn wheat, with AA genome), *Aegilops speltoides* (which contributed to the wheat B genome) and *Aegilops tauschii* (Goatgrass, with DD genome), since they are respectively the immediate diploid sources or closest relatives, carrying the A, B and D genomes of polyploid wheats (Dvorak et al. 1998; Akhunov, Akhunova, and Dvorák 2005).

Triticum genus is made up of diploid wheats, which present the simplest genomic structure corresponding to 2n =14 chromosomes. This group is considered the most ancestral in the evolution of wheat. There are two evolutionary lines of diploid wheats, carriers of two different genomes (named as 'A' genomes), distinguishing the one from *T. urartu* (Einkorn wheat), and the A genome of *T. boeoticum*, which has been proposed as the ancestor of the A genome of *T. monococcum* L. (Golovnina et al. 2007).

Allotetraploid wheats comprise a set of AABB gene pool species and a total of 28 chromosomes. In this group, *T. turgidum* L. stands out, in which we can mention the following subspecies: subsp. *T. turgidum var. durum* (durum wheat), *T. carthlicum*, *T. dicoccum*, and *T. dicoccoides* (Ogbonnaya et al. 2013). These tetraploid wheats come from the hybridization of two wild diploid species, one probably closely to *A. speltoides*. (BB genome) and *T. urartu* (Einkorn wheat, AA genome), (Akhunov, Akhunova, and Dvorák 2005; Jauhar 2006; Petersen et al. 2006). These two species hybridized approximately half a million years ago and produced an ancestral tetraploid hybrid called emmer wheat. It has also been shown that *Ae. tauschii* is the ancestral donor of the D genome to grains which from tetraploids (*T. turgidum*) crossing with *Ae. tauschii spp. strangulata* have become hexaploid (Dvorak et al. 1998). Aaransohn found out the wild progenitor of tetraploid in which contributed to the A and B subgenomes of *T. aestivum* (Aaronsohn et al. 1910). The fertile hybrids between wild emmer wheat (*T. turgidum* ssp*. dicoccoides*), ssp. *durum* and ssp. *dicoccon*, with further information of the full pair of their chromosomes (Sax 1921, 1922) demonstrated that the domesticated tetraploid wheat came from wild emmer and therefore of the A and B subgenomes of *T. aestivum*. Moreover, using whole-genome sequencing the high synteny between ssp*. dicoccoides* and ssp*. durum* have been confirmed (Avni et al. 2017; Maccaferri et al. 2019).

The allohexaploid common bread wheat with D genome (*T. aestivum*, $2n$=AABBDD) is the least diverse genome among the three wheat genomes and certainly less diverse than the diploid ancestor *Ae. tauschii* ($2n = DD$) (Mirzaghaderi and Mason 2019).

Durum wheat (*T. turgidurum* ssp*. durum*) plays a pivotal role in nutrition and is determined not only by the set of nutritional constituents, but also by the versatility in which it is possible to use it in the production of foods that are widespread and particularly appreciated by consumers (Saini, et al. 2022). Einkorn wheat (*T. monococcum* ssp *monococcum*), is a species of ancient cultivation that has played a fundamental role in human nutrition. This specie is tolerant to environmental stress capable of giving an economically viable production in conditions of modest soil fertility (Ullah et al. 2018). The antiquity of Einkorn wheat cultivation has made it a typical crop of certain geographical areas (Brandolini, Volante, and Heun 2016). Domesticated emmer wheat (*T. dicoccum*) with AABB genome (2n = 4x = 28) is the domesticated types of *T. turgidum conv. durum* and *T. turgidum subsp. dicoccum*. The wild type of domesticated emmer wheat is *T. dicoccoides.* Triticale is a hybrid cereal crop between rye (*Secale cereale* L.) and wheat species (AABB or AABBDD) and its productivity and gain quality with hardiness and vigor is typical of wheat, and its high content of lysine is typical of rye (Tyrka and Chełkowski 2004). The tolerance to abiotic stresses and vigorous root system coming from rye, let triticale to grow in light sandy soils with low fertility (Niedziela et al. 2014).

With growing the population and the climate change, there is an increasing demand to find sustainable approaches to control plant pathogens and subsequently improving the crop's yield. Crops have evolved and adapted to cope with most biotic and abiotic stresses, relying on their microbiota to perform many vital biological functions. Some of the processes that involve the microbiota include nutrient acquisition, tolerance to abiotic stress, protection against pathogens, and host immune regulation (Turner, James, and Poole 2013). Knowing the associated microbiota provides the opportunity to achieve an effect on these processes, as well as having an understanding of how plant genotypes have an effect on the colonization of specific microorganisms that would help enhance the beneficial characteristics of the crops (Haney et al. 2015).

In this study, we aimed at understanding the change of soil rhizosphere and root endophytic microbiome communities affected by different factors including varied wheat species and genotypes in two different environments in which one field followed by a minimum tillage management and in the past years had rotation with *Fabaceae* family crops and the other field, on the other hand, was under a conventional agricultural practice and in rotation with *Camelina sativa*.

3.2 Materials and Methods

3.2.1 Plant materials

To perform this experiment, 30 wheat genotypes belonging to six species were selected (Table 3.1). These genotypes were sown in three biological replicates in two fields in Bologna (44°29'38''N; 11°20'34''E; 54 m above sea level) and Foggia (41°27'30.42''N; 15°54'06.77''E; 76m above sea level) at 54 m and 76 m above sea level respectively, which are located in Northern and Southern Italy, respectively. These two locations represent different environments for wheat growth, ie. Northern Italy is typical for bread wheat production whilst the southern climate is a common place for durum wheat cultivation. The agronomical field management in Bologna was under a minimum tillage management and it was in a rotation with *Fabaceae* family crops during the past years. Whilst, the field of Foggia had a conventional agronomical management and in rotation with *Camelina sativa*.

Table 3.1 Details about Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale accessions used in this study.

Genotype	Subspecies	Category	Country of Origin	Mega Environment
Mentana	*aestivum*	BW	ITALY	Southern_Europe
Bologna	*aestivum*	BW	ITALY	Southern_Europe
TDS 281	*dicoccoides*	WEW	TURKEY	Western_Asia
TDS 283	*dicoccoides*	WEW	TURKEY	Western_Asia
TDS 289	*dicoccoides*	WEW	LEBANON	Western_Asia
TDS 263	*dicoccoides*	WEW	LEBANON	Western_Asia
TDS 310	*dicoccoides*	WEW	SYRIA	Western_Asia
TDS 239	*dicoccoides*	WEW	LEBANON	Western_Asia
Molise Colli	*dicoccum*	DEW	ITALY	Southern_Europe
DIC UNIBO-008	*dicoccum*	DEW	ITALY	Southern_Europe
DIC UNIBO-22	*dicoccum*	DEW	UK	Northern_Europe
TDS 231	*dicoccum*	DEW	SYRIA	Western_Asia
DIC UNIBO 54	*dicoccum*	DEW	GERMANY	Western_Europe
DIC UNIBO 49	*dicoccum*	DEW	IRAN	Southern_Asia
Russello SG7	*durum*	DWL	ITALY	Southern_Europe
Kyperounda L28	*durum*	DWL	CYPRUS	Western_Asia
Menceki	*durum*	DWL	TURKEY	Western_Asia
Tetra-IPK 251	*durum*	DWL	ETHIOPIA	Eastern_Africa
EP 4	*durum*	DWL	ETHIOPIA	Eastern_Africa
Cappelli	*durum*	DWL	ITALY	Southern_Europe
Altar 84	*durum*	DWC	CIMMYT	Central_America
Simeto	*durum*	DWC	ITALY	Southern_Europe
Saragolla	*durum*	DWC	ITALY	Southern_Europe
Lloyd	*durum*	DWC	USA	Northern_America
Svevo	*durum*	DWC	ITALY	Southern_Europe
Monastir	*durum*	DWC	FRANCE	Southern_Europe
Monlis	*monococcum*	monococcum	ITALY	Southern_Europe
DV-92	*monococcum*	monococcum	ITALY	Southern_Europe

Quirinale	*triticale*	triticale	ITALY	Southern_Europe
Trica	*triticale*	triticale	ITALY	Southern_Europe

3.2.2 Rhizosphere/bulk soil and root endosphere sampling

A total of 800 rhizosphere/bulk soil and root endosphere samples were collected from 30 genotypes at the first node (Zadoks growth scale 31-33) and beginning of ripening (Zadoks growth scale 87-91) developmental stages in both Bologna and Foggia fields (Figure 3.1). The rhizosphere and bulk soil sampling were carried out following published procedures (Robinson et al. 2021; Kavamura et al. 2019), with partial modifications as summarized here:

1- For every replication, three plants were chosen and pulled out randomly using a sterilized shovel. Plants were then shaked gently to remove the loose soil (top 10 cm of the field) and we considered that as bulk soil. The bulk soil samples of different genotypes within every species were mixed and collected in a Falcon tube and stored in -80 °C prior to processing.

2- The roots which were associated with the remaining soil were placed into a sterile polythene bag, which was shaken vigorously for 30 seconds. The soil that came out in the plastic bag was considered as rhizosphere soil and were collected in Falcon tube and stored in -80 °C prior to processing.

3- The roots of step 2 were collected in Falcon tube and brought back to the laboratory for immediate surface sterilization in which the roots were washed with ethanol 70% for 5 minutes, followed by sterile distilled water for 1 minute, then washed with 2.5% of bleach ($NaClO$) for 5 minutes with agitation, and then washed three more times with sterile distilled water with 1 minute period in each time. After performing all the mentioned steps, using a sterilized scissor the root samples were cut to pieces of 2-3 cm in a clean Petri dish and then collected them in an Eppendorf and store them in -80 °C prior to processing. It should be noted that further samples were stored with glycerol in order to isolate some bacteria from rhizospheric soil and endospheric roots.

After sampling rhizosphere and bulk soil and also root endosphere followed by surface sterilization protocol, all the soil and root samples have been stored in -80 °C until the extraction of the genomic DNA from both soil and root samples.

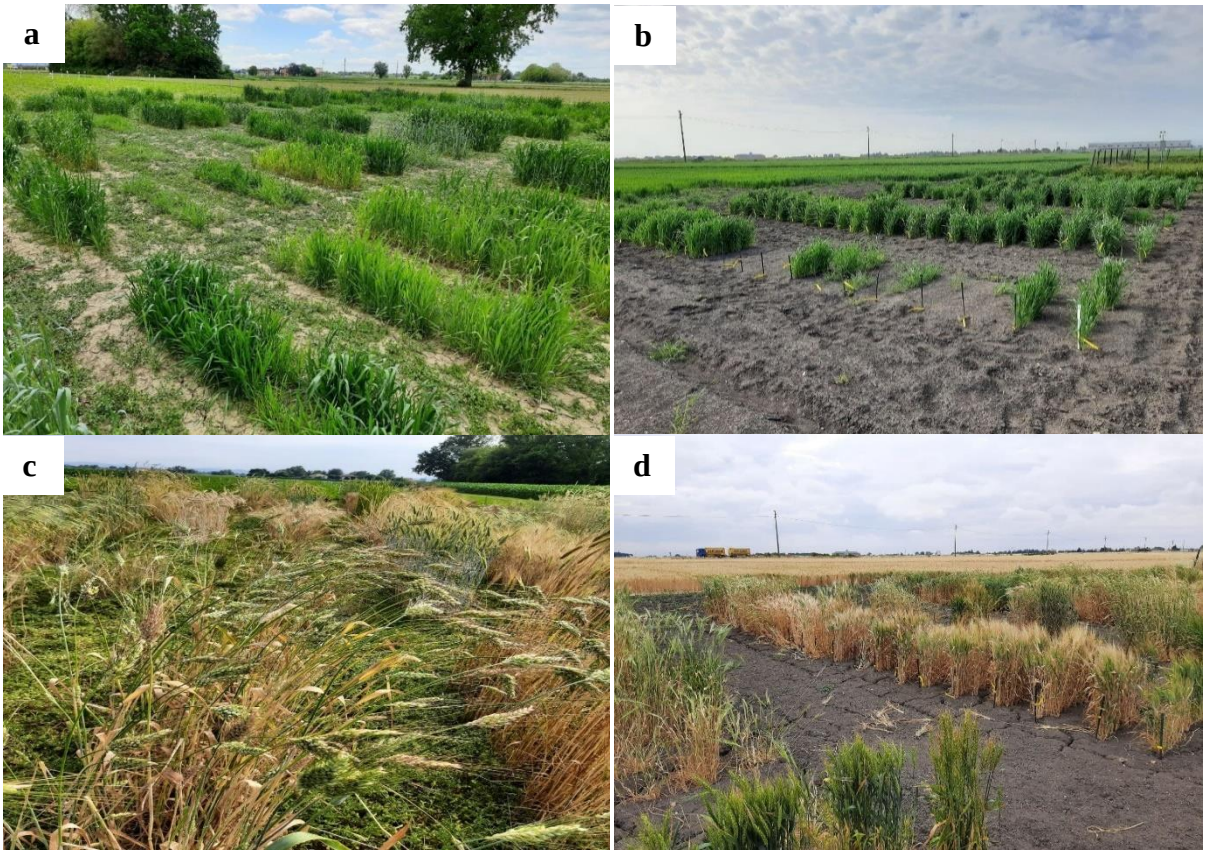

Figure 3.1. a and b) Bologna and Foggia fields, respectively, at the first node development stage; c and d) Bologna and Foggia fields, respectively, at the beginning of ripening stage.

3.2.3 Genomic DNA extraction, 16S rRNA Gene Amplicon Library Preparation from rhizosphere and bulk soil and root endophytic samples in wheat

The DNA preparation from rhizospheric and bulk soil and root samples was carried out using QIAGEN DNeasy PowerSoil Pro kit and QIAGEN DNeasy Plant Mini kit, respectively (Qiagen, Hilden, Germany). The extraction from soil samples followed the guidelines of the manufacturer with a slight modification, i.e. the QIAGEN TissueLyser II have been used instead of horizontal vortex. All the samples were disrupted with QIAGEN TissueLyser II for 4 minutes and 20 seconds with maximum frequency. The root samples were disrupted using a mortar and pestle and then the DNA extraction was carried out based on the guidelines of manufacturer. The quantity and quality of the soil and root DNA were assessed with Nanodrop (Thermo Fisher Scientific, U.S.A.) and then the DNA samples stored at -20 °C. Following quali-quantitative verification, the genomic DNA samples were subjected to PCR for taxonomic informative gene loci. Fragment of the bacterial 16S rRNA gene (V3-V4 region) were amplified with the following primer set: 341F: 5'-CCTACGGGNBGCASCAG -3' and 805R: 5'- GACTACNVGGGTATCTAATCC -3'. Libraries were

prepared in two amplification steps: an initial PCR amplification using locus-specific primers and a subsequent amplification that integrates relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). PNA clamping was applied during the first amplification step to block amplification of host chloroplast and mitochondrial 16S sequences following the manufactures protocol (PNA Bio Inc, Newbury Park, CA).

After PCR amplification and NGS library preparation, sequencing of the samples was carried out on NovaSeq instrument (Illumina, San Diego, CA) using 250-bp paired-end mode. Raw reads were analyzed using QIIME2 - Quantitative Insights into Microbial Ecology (v.2020.8.0), available publicly at http://qiime2.org/ (Bolyen et al. 2019). The quality of raw reads (forward and reverse) have been controlled using MultiQC (v1.14) and followed by DADA2 package in QIIME2 to trim and denoise the low quality reads and removal of the singletons and chimeras (Bokulich et al. 2013; Pauvert et al. 2019; Kuczynski et al. 2011).

3.2.4 Processing of DNA sequence data

All raw FASTQ files for 16S rRNA samples were analyzed and visualized using QIIME 2 (v.2020.8.0) (Bolyen et al. 2019). After importing the data using a Fastq manifest format for paired-end reads, the quality control and denoising were performed using DADA2 package for the rhizospheric and endospheric samples to trim and truncate the low-quality reads and filter out noisy sequences (Callahan et al. 2016). Furthermore, by using DADA2 algorithm the chimeric sequences and singletons were removed. Then, the SEPP function of q2-fragment-insertion plugin took the representative sequences (features) and the reference database (GreenGene v.2013_8) as inputs and returned the phylogenetic tree. Afterwards, the diversity analyses carried out using q2-diversity plugin, which supports computing alpha and beta diversity metrics by applying suitable statistical tests. For alpha diversity, the Shannon index was used to estimate both abundance and evenness of the taxa present for bacterial communities (Callahan et al. 2016; Anderson 2008), the Faith's PD index was used to measure the biodiversity that incorporates phylogenetic differences between species (Faith 1992, 2018), and also the Evenness index was applied to measure of relative evenness of species richness (Jost 2010; Pielou 1966). For the beta diversity (between samples) different metrics were applied such as Bray-Curtis index (a quantitative measure of community dissimilarity), Jaccard index (a qualitative measure of community dissimilarity), unweighted UniFrac index (a qualitative measure of community dissimilarity that incorporates phylogenetic relationships between the features), weighted UniFrac index (a quantitative measure of community dissimilarity that incorporates phylogenetic relationships between the features) for rhizospheric and endospheric samples (Ricotta and Podani 2017; Lozupone et al. 2007; Koeneman and Cavanaugh 2022).

The taxonomic classification was implemented after training the scikit-learn naïve Bayes machine learning classifier for classification of bacterial 16S rRNA marker-gene amplicon sequences (Bokulich et al. 2018). After filtering the biom tables for different taxonomic levels, all the taxonomic bar plots were constructed in R (version 4.0.3).

To test the effect of the different factors on bacterial communities, the Bray-Curtis, Jaccard and weighted UniFrac indices' values were subjected to an Adonis Test (PERMANOVA) (Anderson 2008) in the vegan package in R (version 4.0.3). Principal Coordinate Analysis (PCoA) of Bray-Curtis, Jaccard and weighted UniFrac and unweighted UniFrac indices were carried out in QIIME2.

3.3 Results

3.3.1 Rhizosphere and endophytic microbial diversity in wheat

A total of 444 rhizosphere and bulk soil and 360 root endophytic samples were analyzed to evaluate the bacterial communities using 16S rRNA short-reads-based amplicon sequencing. An average of 1,588,000 and 321,755 fragments (forward and reverse reads) for each soil and root sample of 16S rRNA, was obtained, respectively. After quality filtering and deletion of chimeric and singletons, rarefaction plots using Observed features and Shannon indices showed that stable results for soil samples were reached when approximately > 15,000 and > 5,000 reads per sample in Observed features and Shannon indices were considered, respectively (Figure 3.2).

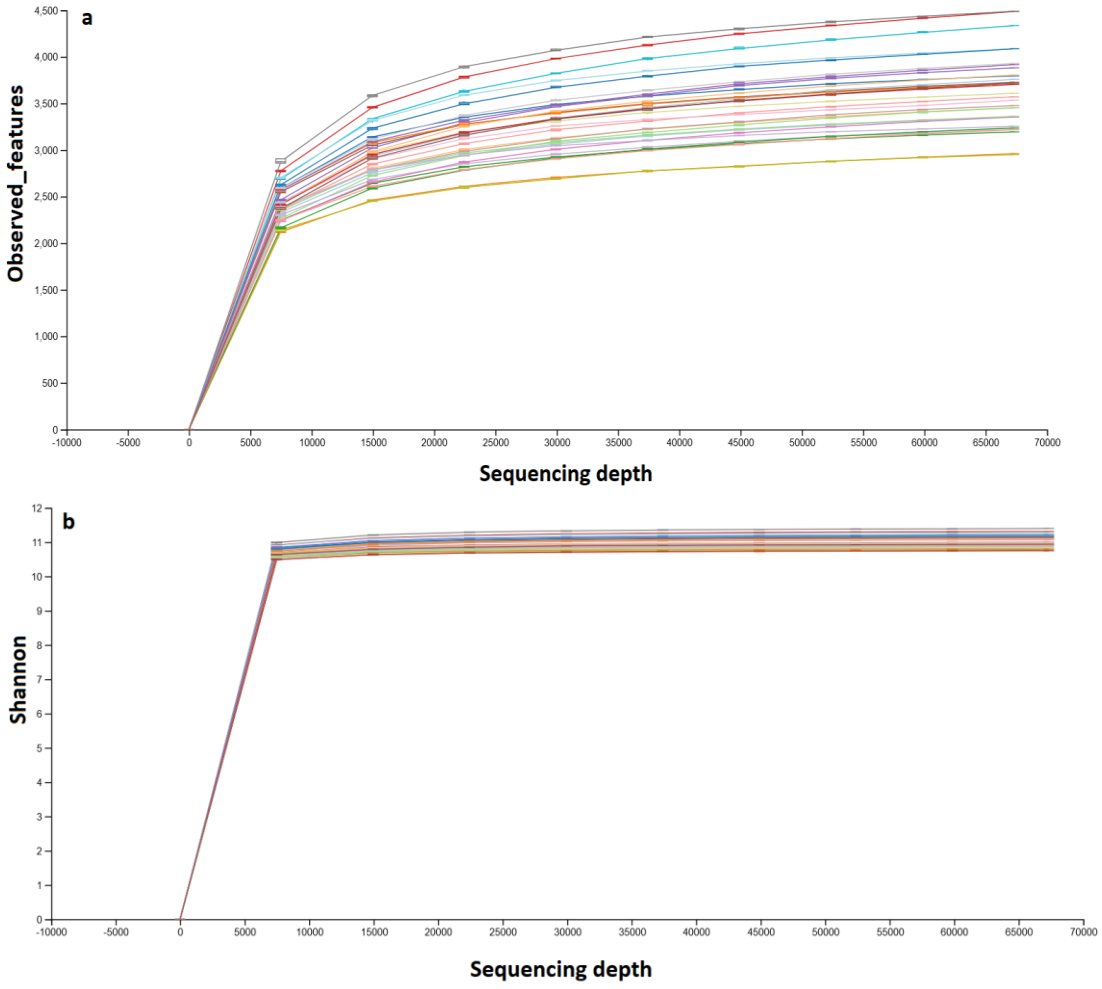

Figure 3.2 Alpha rarefaction plots based on (a) Observed features and (b) Shannon diversity indices from a total of 444 samples of rhizosphere and bulk soil samples from bread wheat, wild emmer wheat, domesticated emmer wheat, durum wheat landraces, durum wheat cultivars, *T. monococcum* and triticale.

The rarefaction plots for endophytic root samples reached to plateau when approximately > 3000 and > 1000 reads per sample in Observed features and Shannon indices were considered, respectively (Figure 3.3). These results supported the robustness of our datasets.

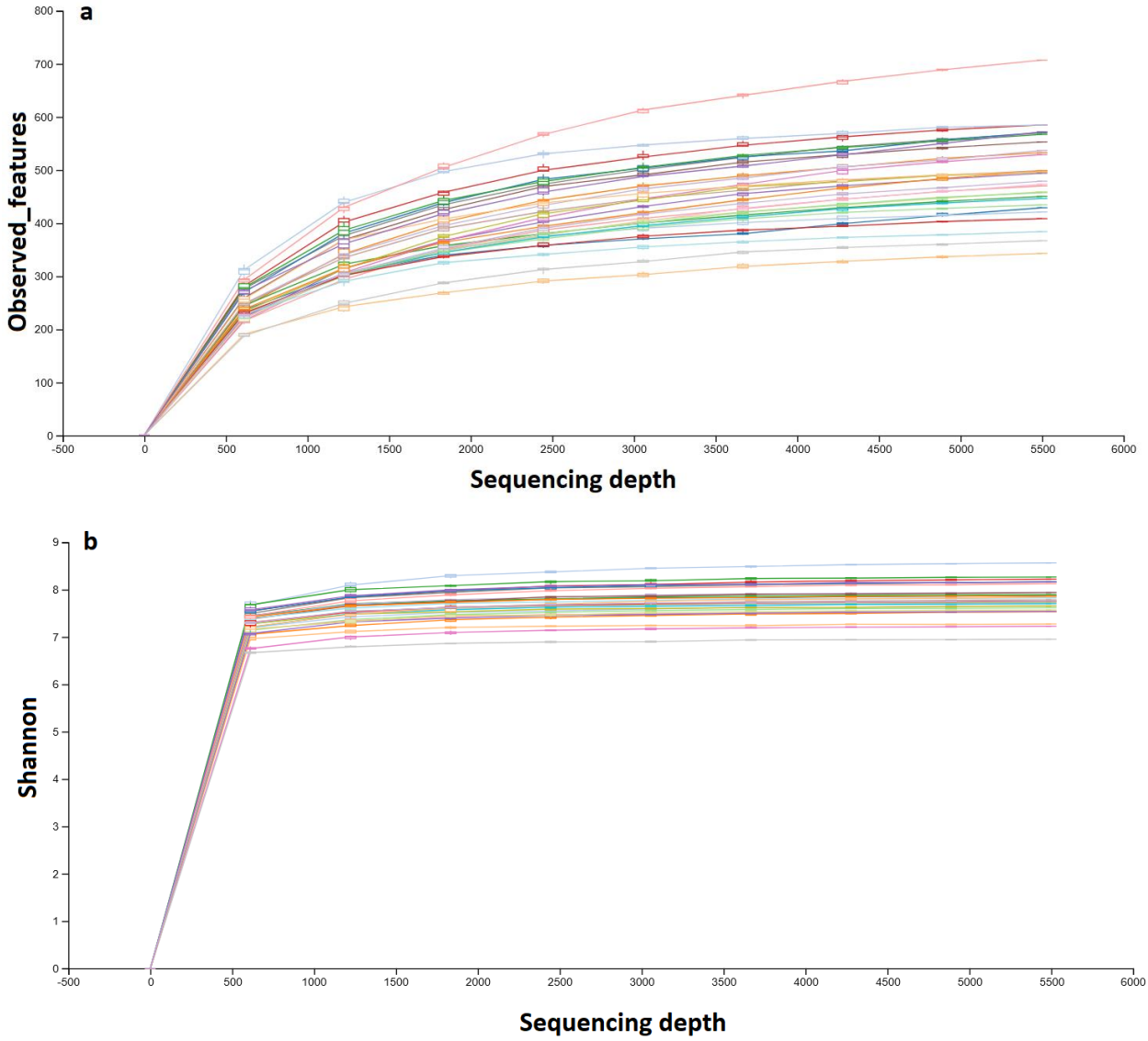

Figure 3.3 Alpha rarefaction plots based on (a) Observed features and (b) Shannon diversity indices from a total of 360 root samples from bread wheat, wild emmer wheat, domesticated emmer wheat, durum wheat landraces, durum wheat cultivars, *T. monococcum* and triticale.

3.3.2 Rhizosphere and endosphere beta diversity using different indices in different wheat species, genotypes and locations

To assess the relative importance of different factors (field, species, cultivar, phenological stage) on microbiome diversities, Bray-Curtis dissimilarity, Jaccard, Unweighted and Weighted UniFrac indices were computed for both rhizospheric and endophytic samples, and PERMANOVA (Adonis Test) and PCoA analyses were carried out based on the corresponding dissimilarity matrices (Figure 3.4 – 3.5). The PERMANOVA analysis showed that the factor of field was the most important factor in structuring bacterial communities using Bray-Curtis ($R^2 = 0.37, P < 0.001$), Jaccard ($R^2 = 0.15, P < 0.001$) and weighted UniFrac ($R^2 = 0.33, P < 0.001$) indices. Moreover, the most important factors in Bologna field using Bray-Curtis index were genotype ($R^2 = 0.15, P < 0.001$), developmental stage ($R^2 = 0.08, P < 0.001$), species ($R^2 = 0.03, P < 0.05$) and soil type ($R^2 = 0.02, P < 0.001$). Whilst, the most important factors in Foggia field were developmental stage ($R^2 = 0.02, P < 0.001$) and soil type ($R^2 = 0.01, P < 0.01$). The main important factors using Jaccard index in Bologna field were genotype ($R^2 = 0.15, P < 0.001$), developmental stage ($R^2 = 0.03, P < 0.001$), species ($R^2 = 0.03, P < 0.05$) and soil type ($R^2 = 0.008, P < 0.001$). As for Foggia field, the important factors using the same index were developmental stage ($R^2 = 0.01, P < 0.001$) and soil type ($R^2 = 0.005, P < 0.01$). The most important factors using weighted UniFrac index in Bologna and Foggia were developmental stage ($R^2 = 0.29, P < 0.001$ and $R^2 = 0.09, P < 0.001$, respectively) and soil type ($R^2 = 0.03, P < 0.001$ and $R^2 = 0.02, P < 0.001$, respectively) (Table 3.2).

Table 3.2 Results of Adonis Test (PERMANOVA) in rhizospheric samples for different factors using Bray-Curtis, Jaccard, weighted UniFrac indices.

Index	Bologna			Foggia		
	Factor	R^2	P_value	**Factor**	R^2	P_value
Bray-Curtis	Genotype	0.15	0.001			
	Developmental stage	0.02	0.001	Developmental stage	0.02	0.001
	Soil type	0.01	0.02	Soil type	0.01	0.01
	Species	0.03	0.03			
Jaccard	Genotype	0.15	0.001			
	Developmental stage	0.03	0.001	Developmental stage	0.01	0.001
	Soil type	0.008	0.001	Soil type	0.005	0.01
	Species	0.03	0.05			
Weighted UniFrac	Developmental stage	0.29	0.001	Developmental stage	0.09	0.001
	Soil type	0.03	0.001	Soil type	0.02	0.001

The PCoA plots of all four indices visualized a good division between Bologna and Foggia rhizospheric soil samples for bacterial communities. The PCoA plot of Bray-Curtis showed the environmental factors (37%) caused the main variation in microbiome diversities. It followed by developmental stage factor (4.7%) and soil type (3.2%). Moreover, the Jaccard PCoA plot showed environmental factors (15.45) caused the main variation in microbiome diversities.

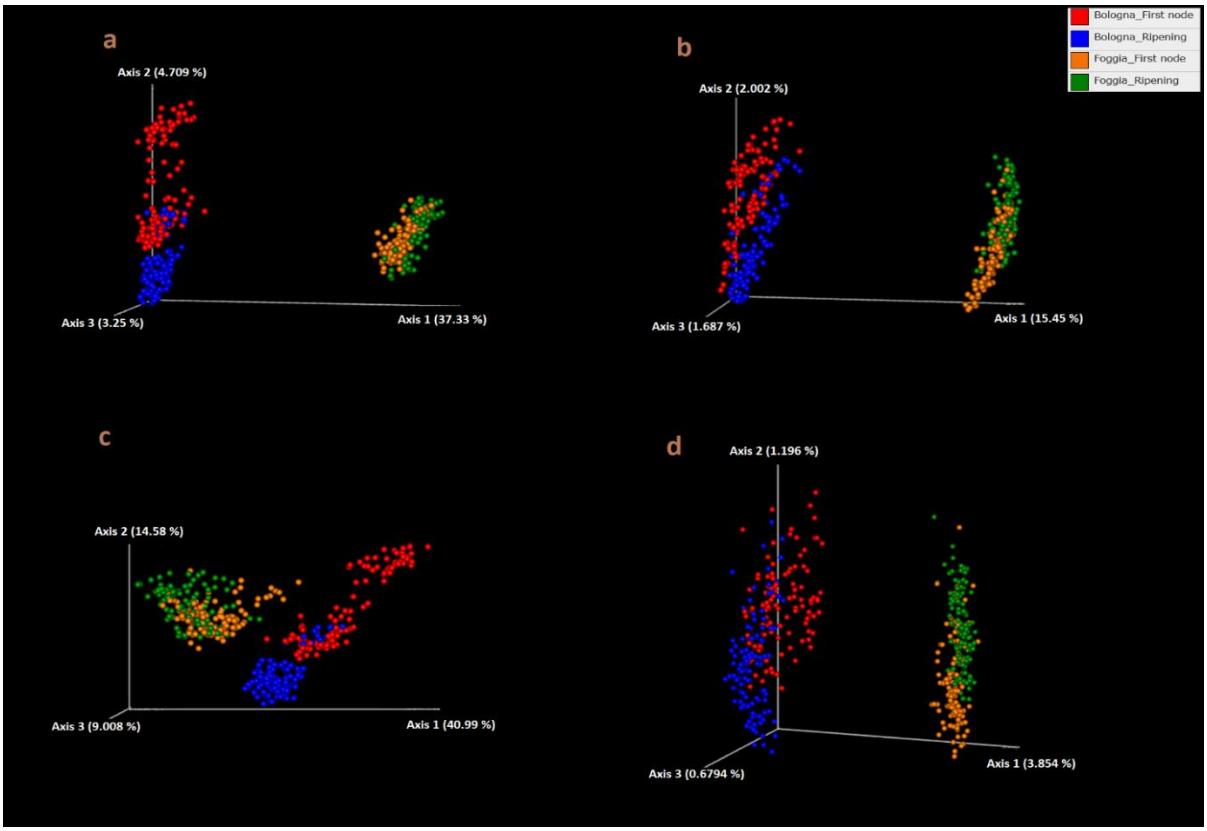

Figure 3.4 Alpha Principal Coordinate analysis (PCoA) plots of soil samples for different wheat species and genotypes in two developmental stages (First node and beginning of ripening) in two fields of Bologna and Foggia based on (a) Bray-Curtis dissimilarity distance; (b) Jaccard distance; (c) Weighted UniFrac; (d) Unweighted UniFrac.

The PCoA plots of all four indices visualized a division between Bologna and Foggia root samples for endophytic bacterial communities. The PCoA plot of Bray-Curtis, Jaccard and weighted UniFrac showed a division between different wheat species in the two fields and some species within each field. The result of Adonis Test (PERMANOVA) for Bray-Curtis showed that the factor of field ($R^2 = 0.11, P < 0.001$), genotype ($R^2 = 0.11, P < 0.05$), species ($R^2 = 0.03, P < 0.001$), developmental stage ($R^2 = 0.06, P < 0.001$) were the most important factors in structuring endophytic bacterial communities. The most important factor in shaping endophytic bacteria using Jaccard distance matrix were genotype ($R^2 = 0.11, P < 0.001$), field ($R^2 = 0.04, P < 0.001$), species ($R^2 = 0.03, P < 0.001$) and developmental stage ($R^2 = 0.02, P < 0.001$) and the

structuring factors using weighted UniFrac were field ($R^2 = 0.11, P < 0.001$), developmental stage ($R^2 = 0.12, P < 0.001$) and species ($R^2 = 0.03, P < 0.05$).

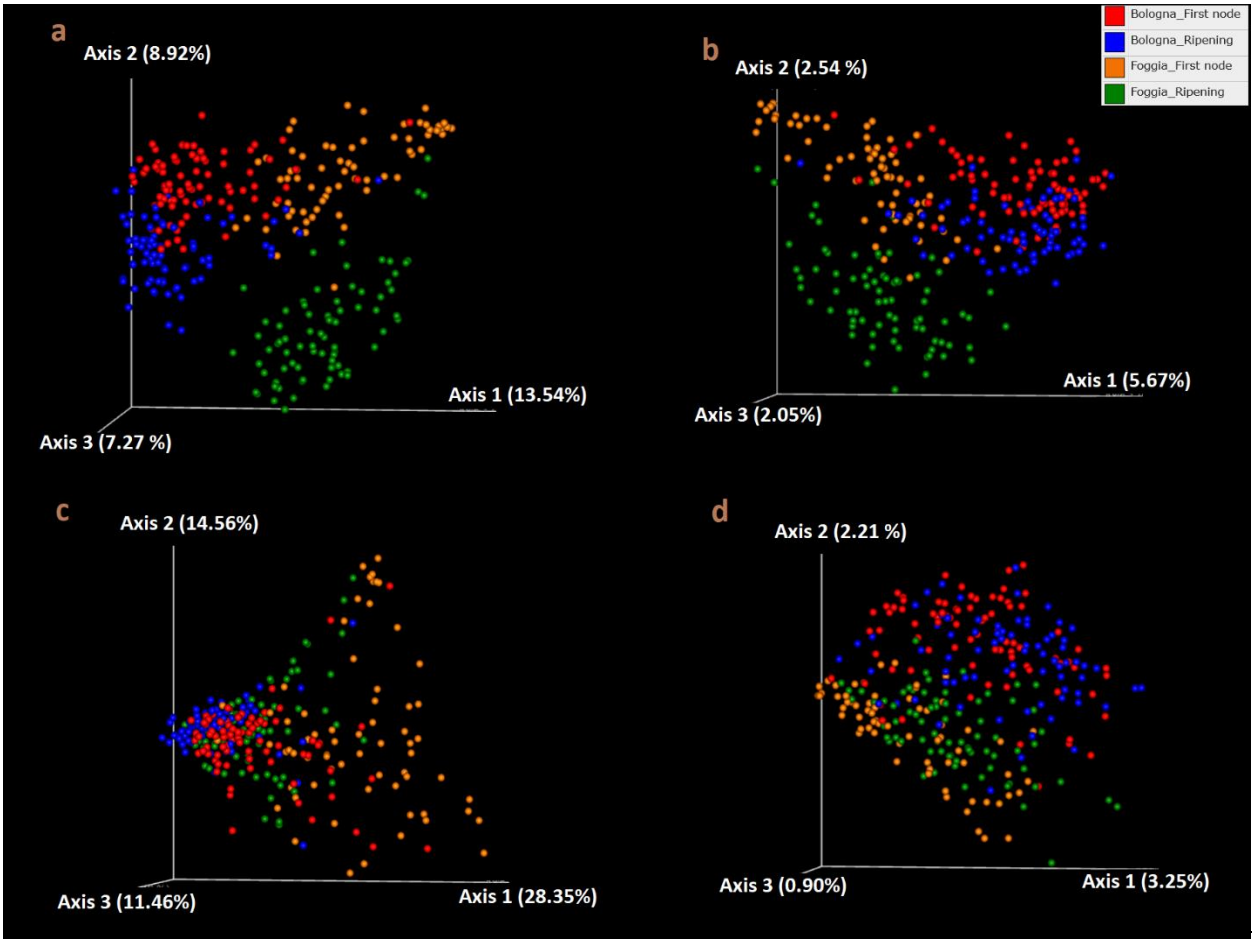

Figure 3.5 Alpha Principal Coordinate analysis (PCoA) plots of root samples for different wheat species and genotypes in two developmental stages (First node and beginning of ripening) in two fields of Bologna and Foggia based on (a) Bray-Curtis dissimilarity distance; (b) Jaccard distance; (c) Weighted UniFrac; (d) Unweighted UniFrac.

3.3.3 Effect of geographical locations on rhizospheric and endospheric microbial (Alpha) diversity using Shannon, Evenness and Faith PD indices

Alpha diversities of the soil and root samples from Bologna and Foggia fields were assessed using Shannon, Evenness (Pielou) and Faith's PD indices. The microbial diversity differed significantly between Bologna and Foggia fields for all three indices (Fig. 3.6). Specifically, Bologna samples always showed higher diversity than Foggia samples. It should be noted that higher diversity entails both higher abundance and evenness of taxa. Specifically, the Shannon index was $M = 11.37$ in Bologna and $M = 10.6$ in Foggia ($adj - P < 0.001. Kruskal - Wallis$ test. KW)); the Evenness index was $M = 0.943$ in Bologna and $M = 0.914$ in Foggia ($adj - P < 0.001. KW$) in Bologna; the Faith's PD was $M = 364.59$ in Bologna and $M = 338.42\ in$ Foggia ($adj - P < 0.01. KW$).

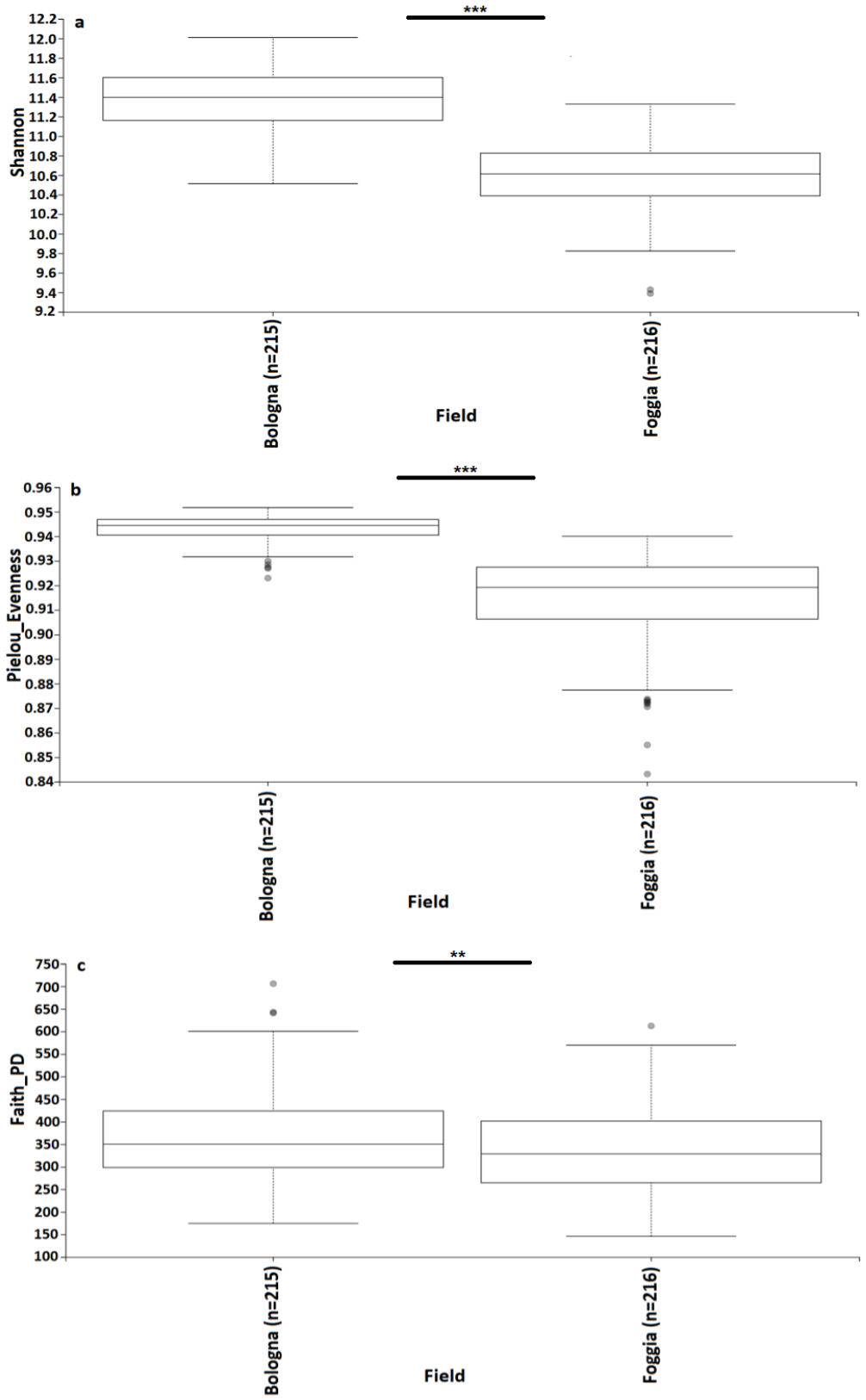

Figure 3.6 Alpha diversity of prokaryotic communities across the two fields of Bologna and Foggia for rhizospheric samples using (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

Similar to rhizospheric result, the root endophytic samples in Bologna had a higher diversity in respect to Foggia using Shannon and Evenness indices (Figure 3.7, Table S3.2). The statistical difference in diversity values for root endophytic samples were assessed using a pairwise Kruskal-Wallis test for comparison of the means of Bologna and Foggia, for three indices (Table S3.2). The mean of alpha diversity using Shannon index in Bologna ($M = 8.36$) was significantly higher than Foggia ($M = 7.15$) field ($adj - P < 0.001$). In addition, the mean of alpha diversity using Evenness index in Bologna ($M = 0.894$) was higher than Foggia ($M = 0.841$) field ($adj - P < 0.001$). However, Bologna and Foggia endophytic samples diversity (M = 95.35 and 98.2, respectively) did not differ when analyzed using Faith's PD index ($adj - P > 0.05. KW$).

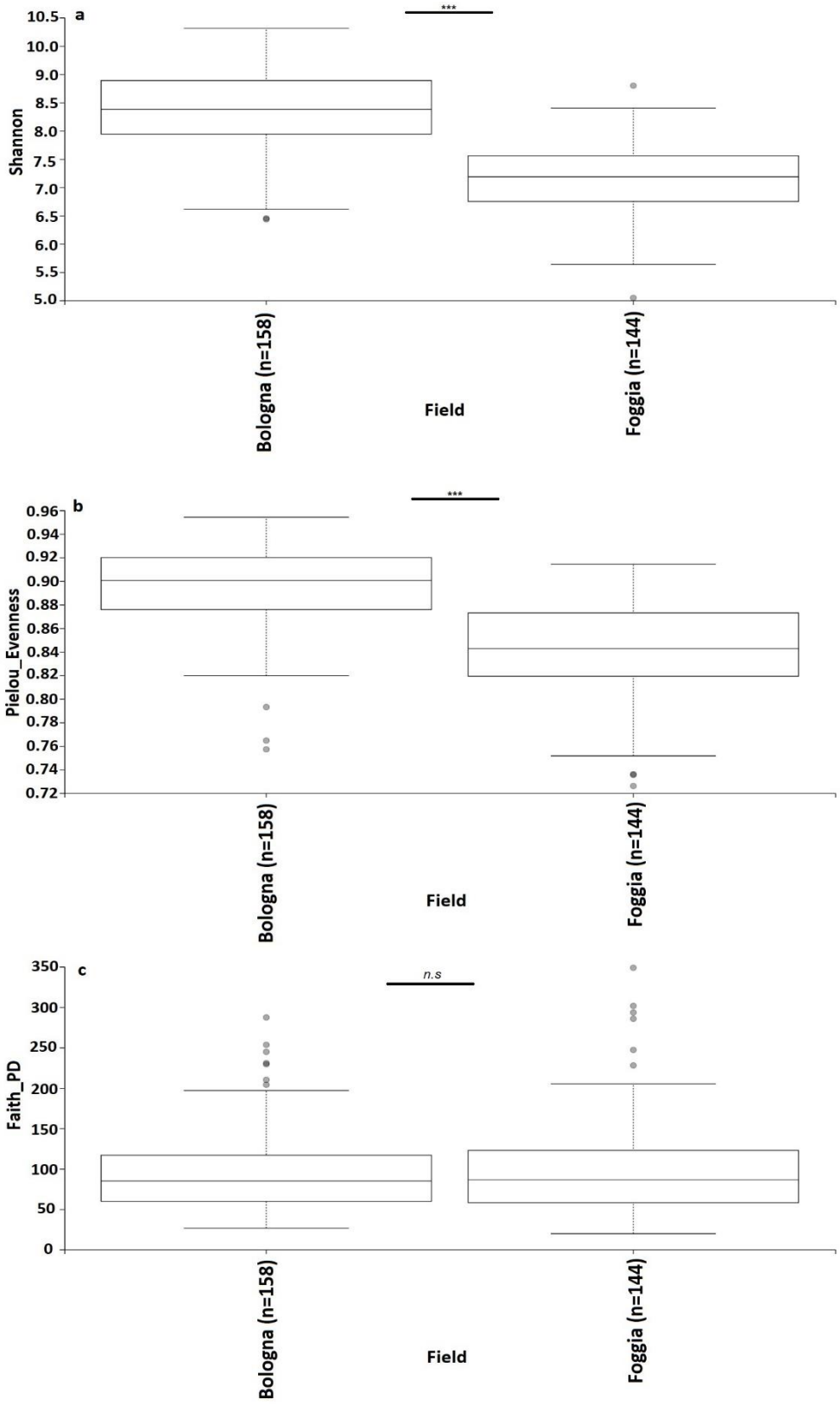

Figure 3.7 Alpha diversity of prokaryotic communities across the two fields of Bologna and Foggia for endospheric samples using, (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

3.3.4 Effect of plant developmental stage on rhizospheric and endospheric microbial (Alpha) diversity using Shannon, Evenness and Faith PD indices

Differences in Alpha diversities of the rhizospheric and endospheric samples across two wheat plants developmental stages, namely 'first node' (Zadoks growth scale 31-33) and 'beginning of ripening' (Zadoks growth scale 87-91) were assessed using Shannon, Evenness (Pielou) and Faith's PD. The microbial diversity in rhizosphere changed significantly between first node and beginning of ripening developmental stages in both fields of Bologna and Foggia (Figure 3.8, Table S3.3). The rhizospheric results of the field of Bologna showed a higher diversity in the beginning of ripening than the first node stage (Shannon index, $M = 11.46$ and $M = 11.27$, respectively, $adj - P < 0.05$). Whilst, we have observed a higher diversity in the first node stage in respect to ripening in Foggia (Shannon index, $M = 10.78$ and $M = 10.42$, respectively, $adj - P < 0.05$) which it could be due to the fact of a very high temperature and consequently environmental differences in Foggia. Furthermore, the alpha diversity using Evenness index for rhizospheric samples in Bologna showed that the microbiome diversity was higher in ripening than the first node developmental stage (Evenness index $M = 0.946$ and $M = 0.940$, respectively, $adj - P < 0.05$). Contrarily, we have seen a different pattern in rhizospheric samples in Foggia in which the microbiome diversity was higher at first node stage than the ripening (Evenness index $M = 0.919$ and $M = 0.910$, respectively, $adj - P < 0.05$). Similar to two previous indices, the microbiome diversity in rhizospheric samples using Faith's PD index was higher in ripening than first node stage in Bologna (Faith's PD index $M = 392.99$ and $M = 335.92$, respectively, $adj - P < 0.05$); and higher in first node than ripening stage in Foggia (Faith's PD index $M = 383.51$ and $M = 294.16$, respectively, $adj - P < 0.05$).

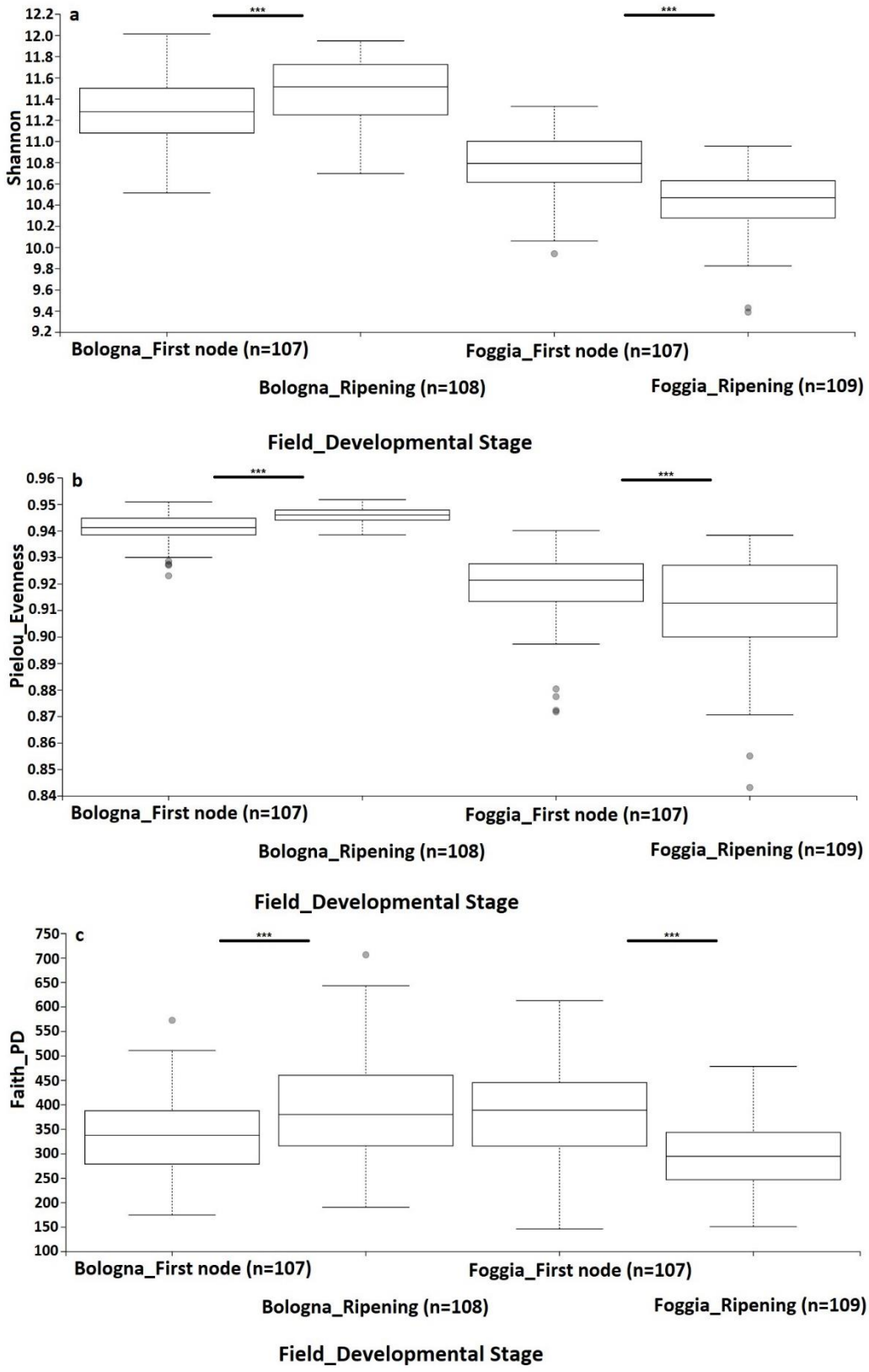

Figure 3.8 Alpha diversity of prokaryotic communities across the two developmental stages (First node and the beginning of ripening) and the two fields of Bologna and Foggia for rhizospheric samples using, (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

Root endophytic samples using Shannon index had a higher diversity of microbial communities in first node stage than beginning of ripening in Bologna field (Shannon index, $M = 8.52$ and $M = 8.17$, $adj - P < 0.05$) but there was no significant change between the two stages in the field of Foggia ($adj - P > 0.05$) (Figure 3.9 and Table S3.4). The alpha diversity using Evenness index for root endophytic samples was showing a higher diversity in first node than ripening stage in Bologna (Evenness index, $M = 0.91$ and $M = 0.87$, respectively, $adj - P < 0.01$) and Foggia (Evenness index, $M = 0.85$ and $M = 0.83$, $adj - P < 0.05$). Similarly, a small but significant higher diversity was observed in first node stage in respect to the beginning of ripening in Bologna (Faith's PD index, $M = 104.11$ and $M = 85.65$, respectively, $adj - P < 0.05$) and Foggia (Faith's PD index, $M = 114.41$ and $M = 84.10$, respectively, $adj - P < 0.05$).

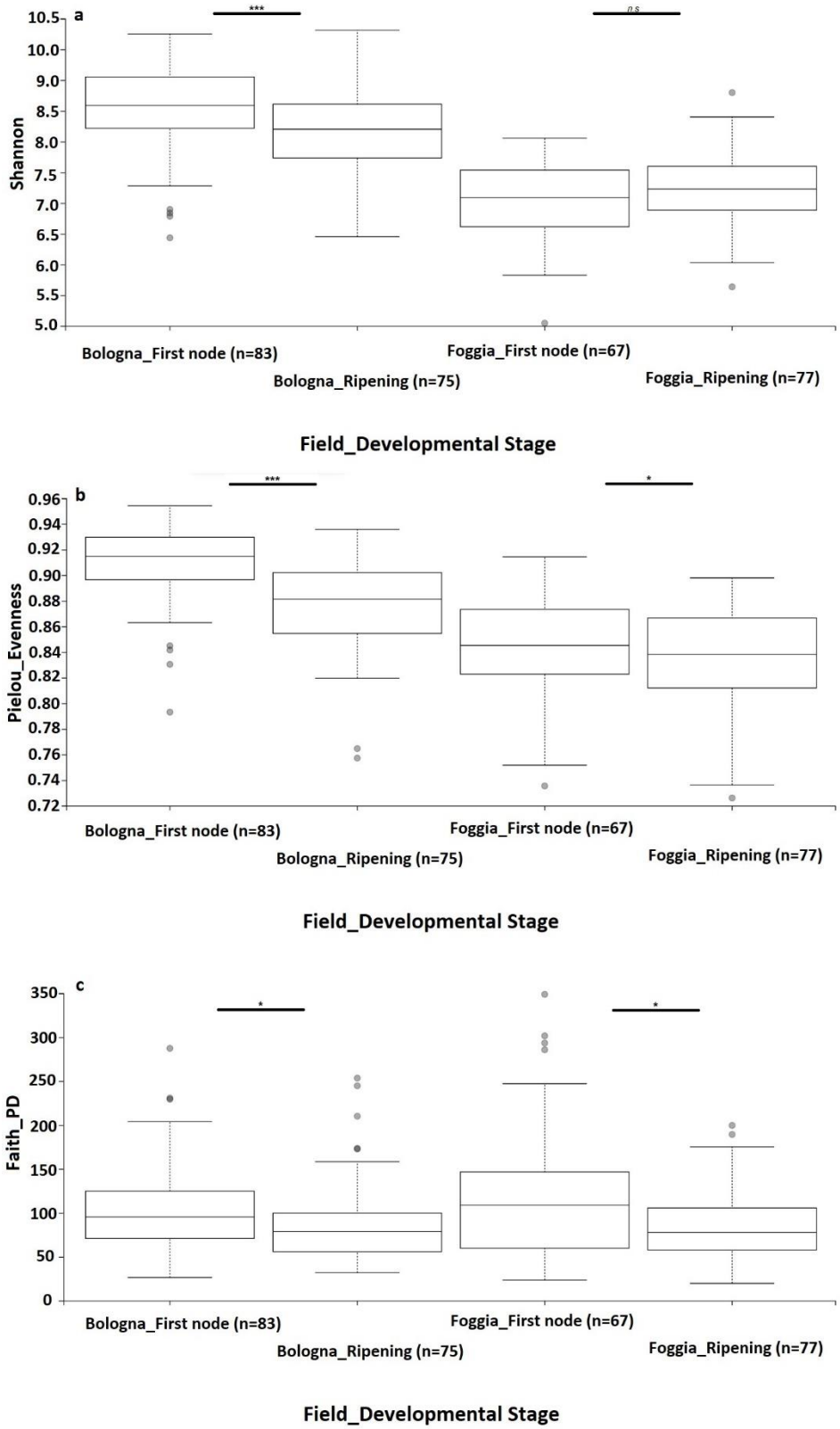

Figure 3.9 Alpha diversity of prokaryotic communities across the two developmental stages (First node and the beginning of ripening) and the two fields of Bologna and Foggia for root endophytic samples using, (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

3.3.5 Effect of wheat species and cultivars on rhizospheric and endospheric microbial (Alpha) diversity using Shannon, Evenness and Faith PD indices

The impact of different species or cultivars on rhizosphere and endosphere diversity was assessed using Shannon, Evenness and Faith's PD indices. We did not see any significant effect due to wheat species in both fields in terms of the biodiversity that incorporates phylogenetic differences between microbiome species (Faith's PD) (Figure 3.10). The pairwise Kruskal-Wallis for all three indices carried out to assess the differences between different pairs of wheat species in both fields (Table S3.5). In majority of cases, there was no significant changes between different wheat species within each field. However, there were some significant changes between different species within each field. Using Shannon index, for instance, there was significant microbiome change between DWC and WEW in Bologna ($M = 11.47\ and\ M = 11.28, \text{respectively}, adj - P < 0.05$). Using Evenness index, the significant differences were between BW and DEW ($M = 0.945\ and\ M = 0.941, \text{respectively}, adj - P < 0.05$), DEW and DWL ($M = 0.941\ and\ M = 0.944, \text{respectively},\ adj - P < 0.05$) and DEW and triticale ($M = 0.941\ and\ M = 0.945, \text{respectively},\ adj - P < 0.05$), all in Bologna. In Foggia there was no significant differences between wheat species (Table S3.5).

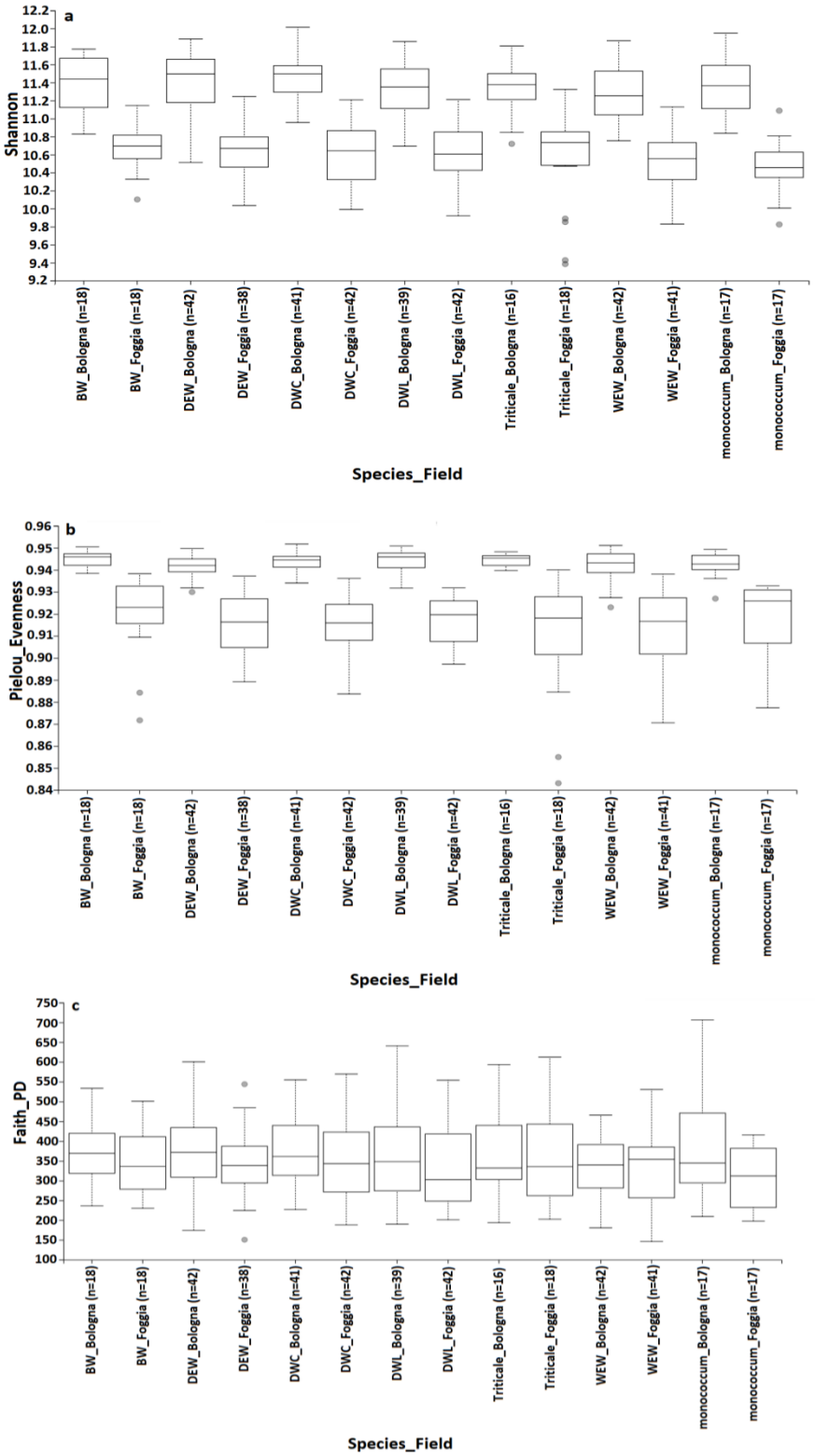

Figure 3.10 Alpha diversity of prokaryotic communities for rhizospheric samples across Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale in two fields of Bologna and Foggia using. (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

In endospheric samples, we did not see any significant difference between wheat species in both fields in terms of the biodiversity that incorporates phylogenetic differences between microbiome species (Faith's PD) (Figure 3.11). The pairwise Kruskal-Wallis for all three indices carried out to assess the different pairs of wheat species in both fields. In majority of cases, there was no significant changes between wheat species within each field. However, there were some significant changes within each field. Using Shannon index, for instance, in Bologna field there was notable differences between DEW and WEW ($adj - P < 0.05$), DEW and *T. monococcum* ($adj - P <= 0.05$), and in Foggia between BW and DEW ($adj - P <= 0.05$), BW and DWC ($adj - P <= 0.01$), BW and DWL ($adj - P <= 0.01$), DWC and *T. monococcum* ($adj - P <= 0.05$), and DWL and *T. monococcum* ($adj - P <= 0.05$). The diversity in Foggia using Evenness index, revealed significant differences between BW and DEW ($adj - P < 0.001$), BW and DWC ($adj - P < 0.01$) and BW and DWL ($adj - P < 0.05$), DEW and *T. monococcum* ($adj - P <= 0.05$). Using the same index, we did not see any notable change in Bologna.

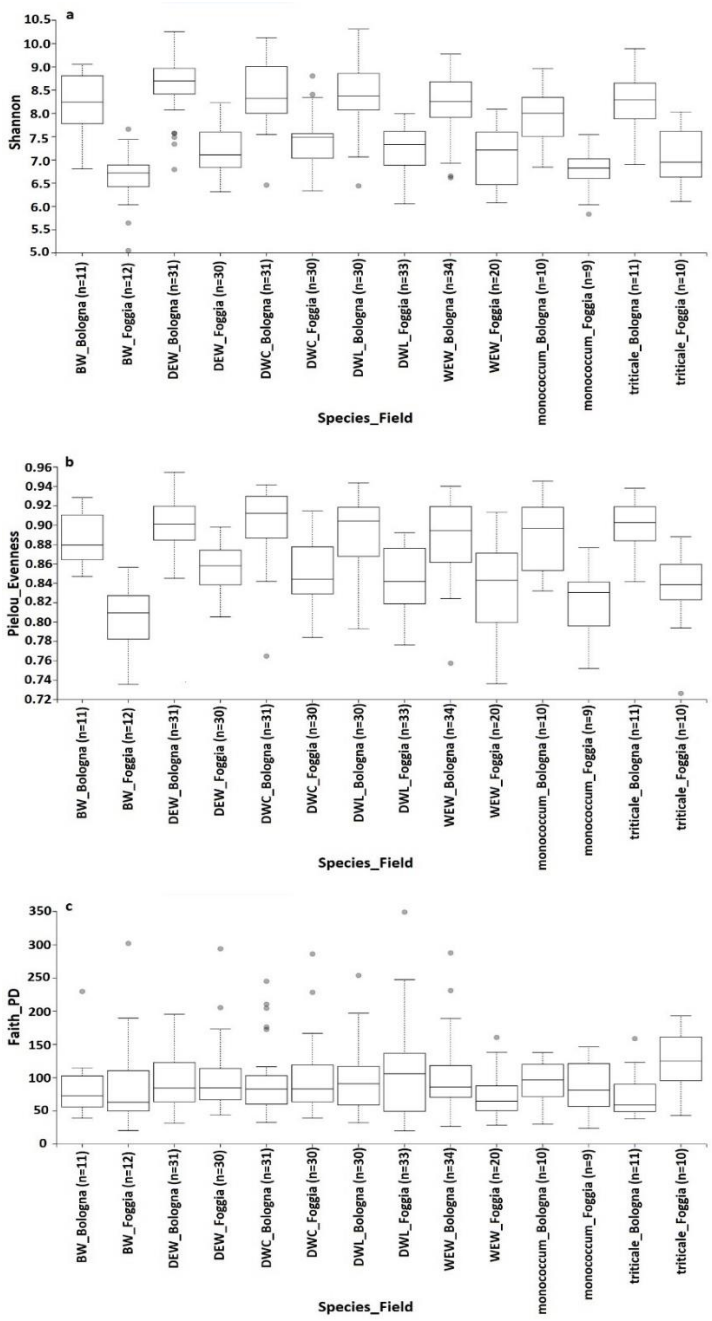

Figure 3.11 Alpha diversity of prokaryotic communities for endophytic samples across Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale in two fields of Bologna and Foggia using. (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

3.3.6 Alpha diversity evaluation in different wheat genotypes for rhizospheric and endospheric samples

The alpha diversity of using three indices of Shannon, Evenness and Faith's PD carried out at genotype level in Bologna and Foggia (Figure 3.12). Using Shannon index there were some genotypes that were significantly different with each other. In Bologna, Altar_84 with Quirinale ($adj - P < 0.05$), Altar_84 with TDS 263 ($adj - P < 0.05$), Altar_84 with Tetra-IPK 251 ($adj - P < 0.05$), DIC UNIBO 49 with Saragolla ($adj - P < 0.05$), DIC UNIBO-008 with DIC UNIBO-22 ($adj - P < 0.05$), DIC UNIBO-22 with Menceki ($adj - P < 0.05$), DIC UNIBO-22 with Monastir ($adj - P < 0.05$), DIC UNIBO 22 with Quirinale ($adj - P < 0.05$), DIC UNIBO 22 with TDS 263 ($adj - P < 0.05$), DIC UNIBO 22 with TDS 310 ($adj - P < 0.05$), DIC UNIBO 22 with Tetra-IPK 251 ($adj - P < 0.05$), Lloyd with TDS 263 ($adj - P < 0.05$), Menceki with Saragolla ($adj - P < 0.05$), Molise Colli with Quirinale ($adj - P < 0.05$), Molise Colli with TDS 263 ($adj - P < 0.05$), Molise Colli with TDS 310 ($adj - P < 0.05$), Molise Colli with Tetra-IPK 251 ($adj - P < 0.05$), Monastir with Saragolla ($adj - P < 0.05$), Quirinale with Russello SG7 ($adj - P < 0.05$), Quirinale with Saragolla ($adj - P < 0.05$), Russello SG7 with TDS 263 ($adj - P < 0.05$), Saragolla with TDS 263 ($adj - P < 0.05$), Saragolla with TDS 310 ($adj - P < 0.05$), Saragolla with Tetra-IPK 251 ($adj - P < 0.05$), Saragolla with Bologna ($adj - P < 0.05$), Simeto with Bologna ($adj - P < 0.05$), Svevo with TDS 263 ($adj - P < 0.05$) were significantly different with each other. In Foggia there was no significant change between the genotypes using Shannon index.

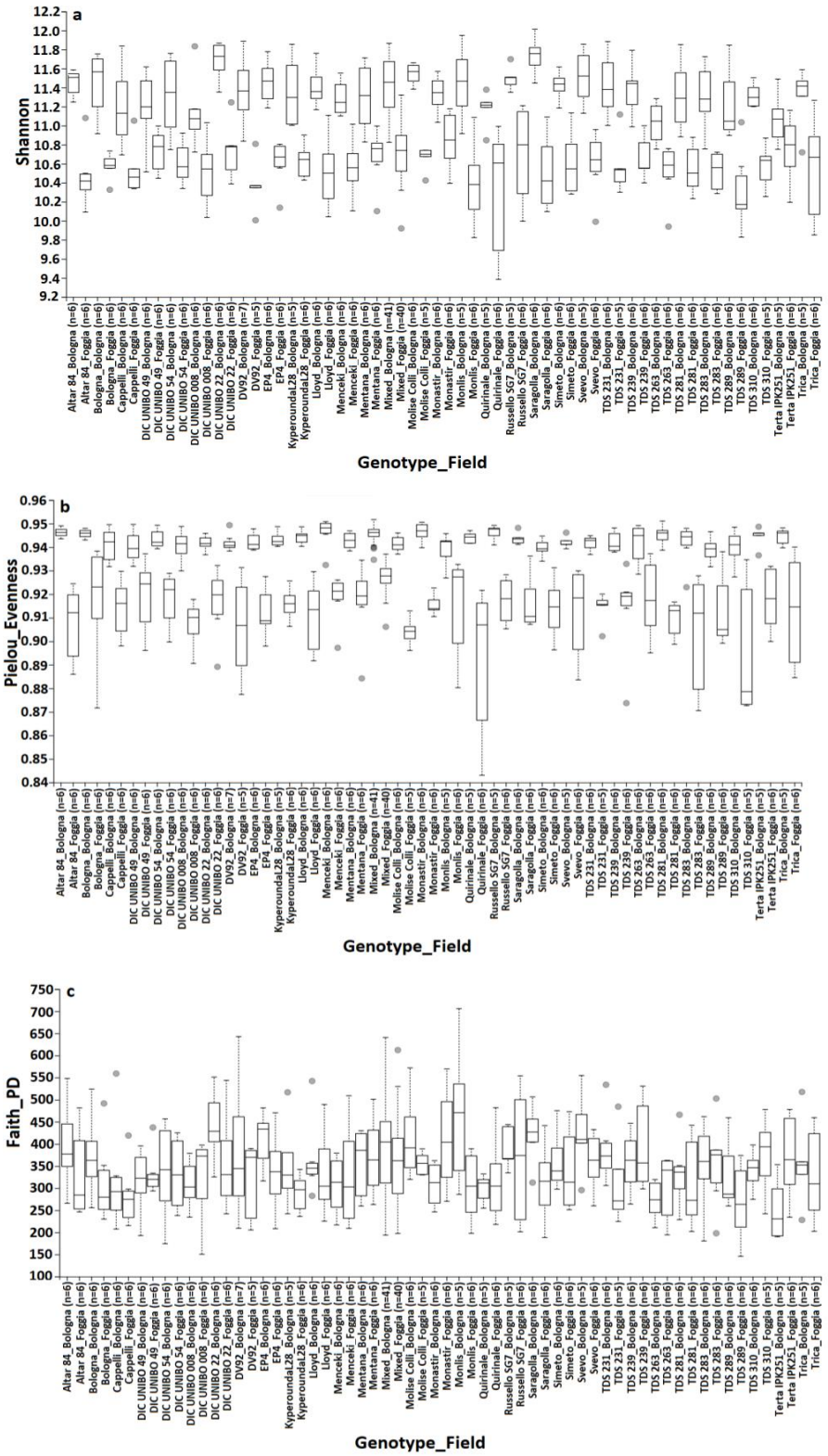

Figure 3.12 Alpha diversity of prokaryotic communities for rhizospheric samples across different wheat genotypes in two fields of Bologna and Foggia using. (a) Shannon index; (b) Evenness index; (c) Faith's PD index.

3.3.7 Analysis of taxonomic distribution of rhizospheric and endophytic samples

The taxonomic distributions of rhizospheric bacterial communities for two fields, species and genotypes are given in Figure 3.13 at the phylum taxonomic level. Only groups with relative abundances $\geq 1.0\%$ were reported. As far as bacteria were concern, the first, second and third most frequent phyla in Bologna field were *Actinobacteria* (28.02%), *Proteobacteria* (19.59%) and *Planctomycetes* (13.70%), respectively. As for Foggia field, the first, second and third most frequent phyla were *Actinobacteria* (35.65%), *Proteobacteria* (21.61%) and *Chloroflexi* (12.02%) (Figure 3.13). A relatively large portion of bacterial sequences in both Bologna and Foggia fields (approx. 17%) remained unassigned (Figure 3.13).

At the family level, the most frequent bacterial taxa across different wheat species and both fields in rhizospheric samples were *Geodermatophilaceae*, *Microbacteriaceae, Nocardioidaceae, Micromonosporaceae, Propionibacteriaceae, Streptomycetaceae* and *C111* (*Actinobacteria*), *Dolo 23* and *Kouleothrixaceae* (*Chloroflexi*), *Isosphaeraceae* (*Planctomycetes*), *Methylobacteriaceae, Rhodospirillaceae* and *Sphingomonadaceae* (*Proteobacteria*) (Table 3.3).

Similarly, the taxonomic distributions of endophytic bacterial communities for two fields and species are given in Figure 3.14 at the phylum taxonomic level. Like before, only groups with relative abundances $\geq 1.0\%$ were reported. As far as bacteria were concern, the first, second and third most frequent phyla in Bologna and Foggia were *Proteobacteria* (38.06% and 46.73%, respectively), *Actinobacteria* (36.58% and 38.76%, respectively) and *Saccharibacteria* (8.31% and 5.94%, respectively) (Figure 3.14). At the family level, the most frequent bacterial taxa across different wheat species and both fields in endophytic root samples were *Actinosynnemataceae*, *Microbacteriaceae, Streptomycetaceae, Nocardioidaceae, Micrococcaceae* and *Micromonosporaceae* (*Actinobacteria*), *Rhizobiaceae, Sphingomonadaceae*, *Mitochondria* and *Caulobacteraceae* (*Proteobacteria*) (Table 3.4).

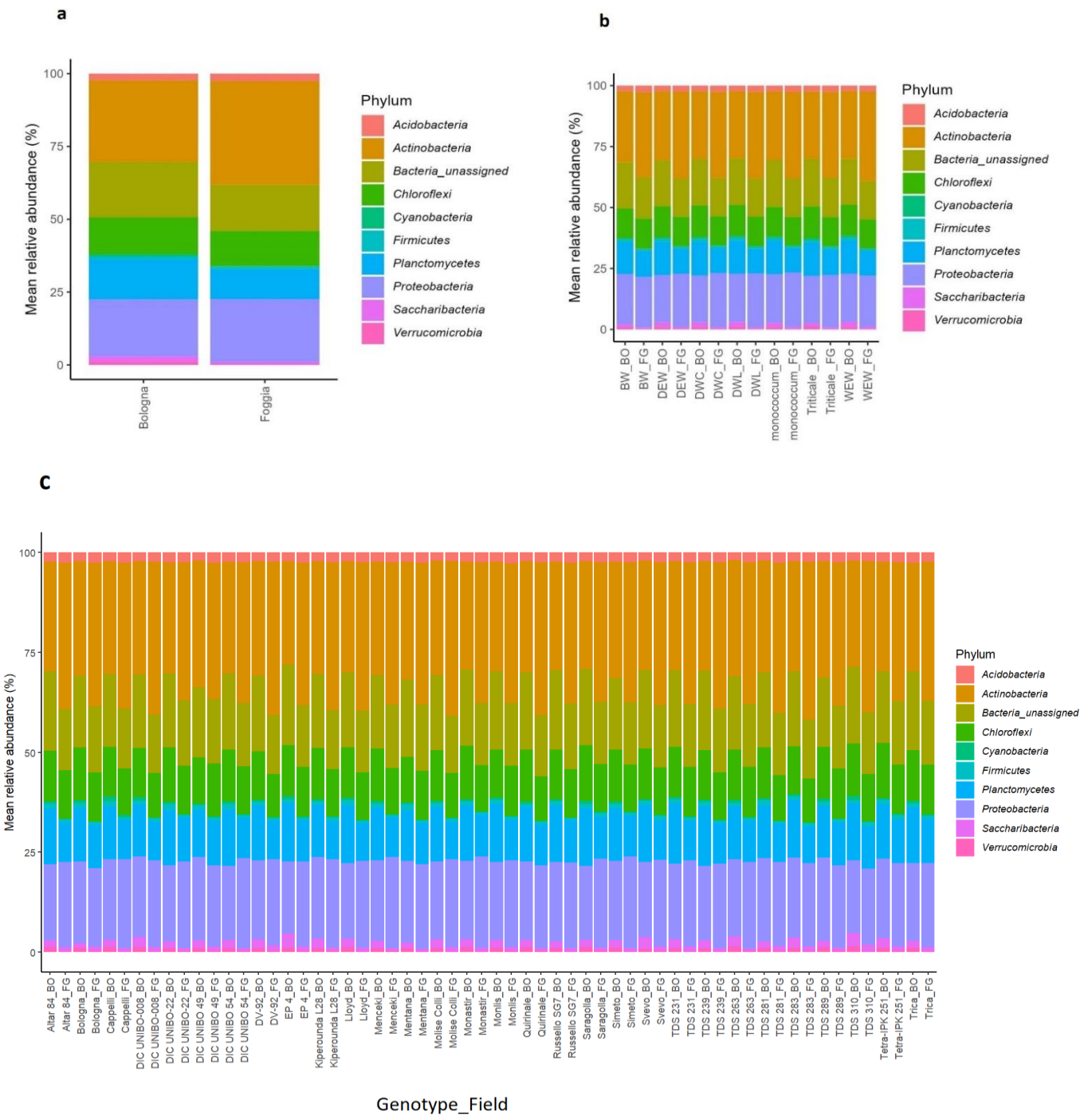

Figure 3.13 Taxonomy assignment bar plots at phylum level for (a) two fields of Bologna and Foggia, (b) Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale in the fields of Bologna (BO) and Foggia (FG), (c) different genotypes in Bologna (BO) and Foggia (FG) fields.

Table 3.3 Percentages of identified bacterial at family level from rhizosphere region in two fields of Bologna (BO) and Foggia (FG) in Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale represented in more than 50% of the samples.

	Abundance % (*)													
Family	**BW_BO**	**BW_FG**	**DEW_BO**	**DEW_FG**	**DWC_BO**	**DWC_FG**	**DWL_BO**	**DWL_FG**	**Monococcum_BO**	**Monococcum_FG**	**WEW_BO**	**WEW_FG**	**Triticale_BO**	**Triticale_FG**
Microbacteriaceae	4.11%	10.40%	4.90%	11.77%	4.33%	11.37%	4.51%	11.14%	4.45%	11.23%	5.16%	13.00%	3.85%	11.45%
Geodermatophilaceae	1.30%	2.38%	1.14%	2.44%	1.08%	2.48%	1.12%	2.47%	1.15%	2.68%	1.06%	2.34%	1.14%	2.68%
C111	1.62%	1.46%	1.56%	1.40%	1.57%	1.39%	1.49%	1.41%	1.53%	1.40%	1.49%	1.43%	1.59%	1.47%
Micromonosporaceae	2.55%	2.26%	2.18%	2.12%	2.17%	2.10%	2.14%	2.16%	2.21%	2.23%	1.93%	2.00%	2.23%	2.13%
Nocardioidaceae	3.09%	2.80%	3.09%	3.04%	3.01%	3.05%	3.02%	3.14%	2.97%	2.87%	2.95%	3.02%	3.05%	2.87%
Dolo 23	1.11%	1.10%	1.18%	1.03%	1.17%	1.03%	1.12%	1.00%	1.13%	1.02%	1.14%	1.00%	1.20%	1.04%
Isosphaeraceae	2.58%	1.26%	2.64%	1.11%	2.75%	1.12%	2.62%	1.12%	2.78%	1.11%	2.61%	1.15%	2.75%	1.23%
Propionibacteriaceae	1.17%	1.89%	1.16%	1.80%	1.12%	1.84%	1.14%	1.80%	1.23%	1.94%	1.13%	1.74%	1.13%	1.81%
Streptomycetaceae	1.26%	2.61%	1.15%	2.38%	1.21%	2.34%	1.23%	2.39%	1.41%	2.51%	1.15%	2.21%	1.24%	2.39%
Kouleothrixaceae	1.79%	1.80%	1.88%	1.87%	1.94%	1.83%	1.88%	1.85%	1.75%	1.89%	1.77%	1.79%	1.82%	1.79%
Methylobacteriaceae	1.67%	2.27%	1.58%	2.54%	1.46%	2.56%	1.61%	2.45%	1.54%	2.52%	1.63%	2.35%	1.52%	2.44%
Rhodospirillaceae	5.07%	4.65%	4.39%	5.08%	4.35%	5.24%	4.44%	5.12%	4.50%	5.52%	4.08%	4.65%	4.38%	5.17%
Sphingomonadaceae	1.56%	2.00%	1.60%	2.24%	1.45%	2.15%	1.61%	2.16%	1.62%	2.05%	1.69%	1.94%	1.56%	2.11%

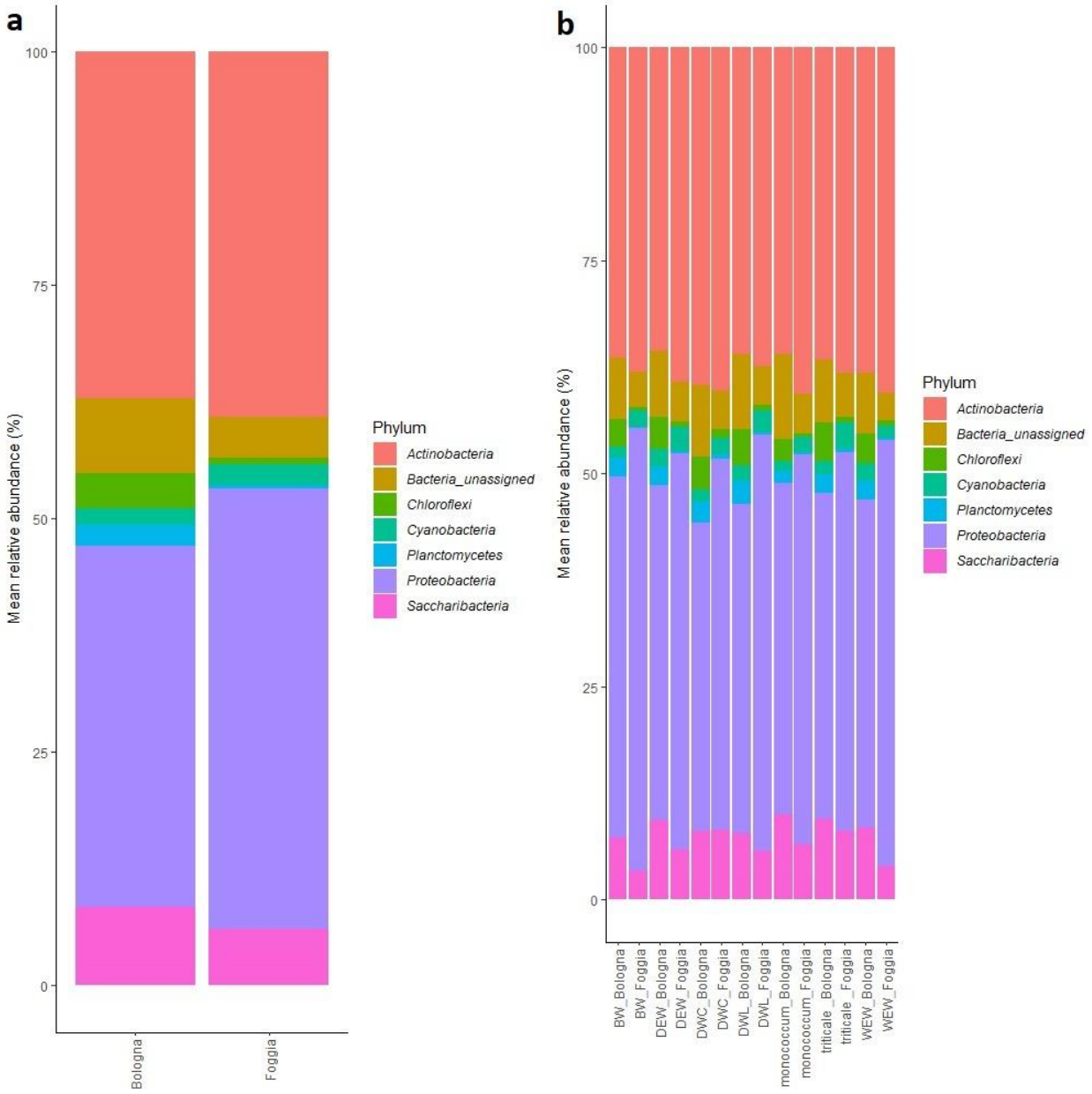

Figure 3.14 Taxonomy assignment bar plots of root endophytic samples at phylum level for (a) two fields of Bologna and Foggia, (b) Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale in the fields of Bologna (BO) and Foggia (FG).

Table 3.4 Percentages of identified bacterial at family level from root endosphere in two fields of Bologna (BO) and Foggia (FG) in Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale represented in more than 50% of the samples.

	Abundance % (*)													
Family	**BW_BO**	**BW_FG**	**DEW_BO**	**DEW_FG**	**DWC_BO**	**DWC_FG**	**DWL_BO**	**DWL_FG**	**Monococcum_BO**	**Monococcum_FG**	**WEW_BO**	**WEW_FG**	**Triticale_BO**	**Triticale_FG**
Actinosynnemataceae	10.24%	17.09%	10.48%	17.40%	12.19%	16.16%	10.04%	16.64%	10.37%	23.45%	13.63%	21.41%	12.48%	20.23%
Rhizobiaceae	11.83%	10.50%	9.48%	9.84%	9.75%	8.18%	10.06%	9.71%	10.61%	9.54%	10.39%	8.96%	8.60%	10.07%
Sphingomonadaceae	5.57%	10.73%	4.04%	8.23%	3.77%	8.31%	4.21%	10.36%	4.12%	5.25%	4.18%	14.41%	6.56%	7.25%
Mitochondria	6.13%	9.58%	4.50%	8.42%	3.47%	6.69%	3.89%	7.76%	5.40%	14.78%	3.77%	3.55%	3.45%	10.68%
Microbacteriaceae	6.21%	1.45%	5.22%	2.10%	4.88%	2.13%	5.39%	1.95%	4.66%	1.59%	5.57%	2.41%	4.32%	1.93%
Streptomycetaceae	3.29%	7.62%	2.70%	6.03%	2.93%	6.12%	2.89%	5.53%	5.94%	4.96%	2.14%	3.57%	2.65%	4.20%
Caulobacteraceae	2.74%	5.56%	2.51%	2.89%	2.29%	3.52%	2.18%	3.97%	2.73%	1.55%	2.19%	6.51%	3.79%	2.42%
Nocardioidaceae	2.68%	1.45%	3.41%	2.62%	3.75%	2.70%	3.31%	2.25%	2.94%	1.18%	2.81%	1.75%	3.12%	2.35%
Micrococcaceae	1.97%	2.81%	1.78%	2.98%	2.27%	3.98%	1.67%	3.55%	1.16%	3.37%	1.64%	3.80%	1.49%	2.01%
Micromonosporaceae	2.18%	1.27%	2.20%	1.81%	3.02%	2.34%	2.41%	1.56%	2.29%	1.11%	2.28%	1.57%	3.35%	1.83%

3.4 Discussion and conclusion

In this study we characterized the rhizospheric and endophytic prokaryotic microbial communities associated to different wheat species and genotypes grown in two fields with different agricultural managements, and we tested for the effect of major factors such as field, wheat species, wheat genotypes, and phenological stages on microbial diversity.

Agricultural practices and management are one of the main factors affecting microbiome diversity whether in the rhizospheric soil or as root endophytes (Carbonetto et al. 2014; Debenport et al. 2015; Reganold and Wachter 2016; Hartman et al. 2017, 2018). Land management was clearly shown to have a strong impact on shaping microbiome communities (Hartman et al. 2017, 2018; Kraut-Cohen et al. 2020; Peltoniemi et al. 2021) in which crops with rotation and minimum tillage have higher diversity in respect to monoculture farming (Bennett et al. 2012; Hilton et al. 2013; Santhanam et al. 2015; J. Zhang et al. 2021). In accordance with the above studies, in our experiment we found out that the rhizospheric and endophytic microbiome communities were more diverse in Bologna field, characterized by minimum tillage management and rotation with other crops (*Fabaceae*) in respect to Foggia field that was cultivated under a conventional management.

Other factors under our investigation were plant species and genotypes in changing microbiome communities. Previous studies showed that the rhizospheric microbiome changes through different plant species. For instance, peas (legumes) have higher microbiome diversities than wheat and oats (cereals) due to their interaction with nitrogen fixing rhizobacteria (Turner, James, and Poole 2013; Turner et al. 2013; Ding, Palmer, and Melcher 2013; Bouffaud et al. 2014; Ding and Melcher 2016). In similar patterns with these studies, we have seen significant rhizospheric microbiome changes between different wheat species within each field. Furthermore, our results showed that wheat genotype was another important factor in changing the rhizospheric microbiome communities which it was in accordance with previous studies (Peiffer et al. 2013; Afzal et al. 2019; Zhong et al. 2019; Singh et al. 2020; R. Gupta et al. 2021). We have also seen in endophytic samples that wheat species and genotypes within each field was influencing the bacterial endophytes, which supports previous findings (Andreote et al. 2010; Hardoim et al. 2015; Hirsch and Mauchline 2012; Govindasamy et al. 2017).

Previous studies had shown the importance of age or developmental stage of the plant on influencing the microbiome communities in *Arabidopsis* (Micallef et al. 2009; Yuan et al. 2015), *Medicago* (Mougel et al. 2006), maize (Baudoin, Benizri, and Guckert 2002; Wattenburger, Halverson, and Hofmockel 2019; Xiong et al. 2021), pea (Houlden et al. 2008), sugar beet (Houlden et al. 2008) and wheat (Houlden et al. 2008; Robinson et al. 2016; Gdanetz and Trail 2017). In line with these studies, our results showed that the bacterial microbiome communities change through the developmental stages in wheat in both rhizospheric and endophytic

samples, although this occurred in currently unexpected ways. Specifically, the prokaryotic rhizospheric alpha diversity at the beginning of ripening developmental stage (Zadoks growth scale 87-91) in Bologna was higher prokaryotic than at the first node stage (Zadoks growth scale 31-33). Unexpectedly, this pattern was inverse in Foggia. One possible explanation was the very different environmental conditions between Bologna and Foggia, specifically at the second sampling (ripening). At this time, soil moisture and air temperature in Foggia were already typical of full summer, which is overall rather extreme when compared to Bologna, thus likely driving the soil microbial communities to more specialized and less diverse biomes.

Another aim of our experiment was to understand the main factors that shape the bacterial communities in rhizospheric and endospheric samples. We found out that field location was the most important factor in shaping the microbiome communities using Bray-Curtis (37%), Jaccard (15%) and weighted UniFrac (33%) indexes. The most important factors using Bray-Curtis index in the field of Bologna were genotype (15%), species (3%), developmental stage (2%) and soil type (1%). This was in accordance with previous studies that found these factors have major effect on the microbiome communities (Navrátilová et al. 2019; Berlanas et al. 2019; Wright et al. 2022). Whilst, the important factors in the field of Foggia were developmental stage (2%) and soil type (1%) but we did not find significant effect between species and genotypes.

Our taxonomical assignments for rhizosphere samples showed that *Actinobacteria* (28.02%), *Proteobacteria* (19.59%) and *Planctomycetes* (13.70%) were dominant phyla in Bologna. The dominant rhizospheric bacterial phyla in Foggia were similar, with *Actinobacteria* (35.60%), *Proteobacteria* (21.61%) and *Chloroflexi* (12.02%). Furthermore, we have observed these bacterial phyla in all wheat species and genotypes samples in Bologna and Foggia. The prevalence of *Proteobacteria* and *Actinobacteria* in the rhizosphere was previously observed in similar studies in *Vitis vinifera* (Vega-Avila et al. 2015; Zarraonaindia et al. 2015; Wright et al. 2022) and *Arabidopsis thaliana* (Lundberg et al. 2012).

Previous studies showed that *Proteobacteria* and *Actinobacteria* were two of the dominant phyla for endophytic assemblies in wheat (Hardoim et al. 2015; Robinson et al. 2016), barley (Rahman et al. 2018), maize (L. Zhang et al. 2022) and grapevine (Zarraonaindia et al. 2015; Wright et al. 2022). Accordingly, in our experiment, the most frequent phyla in root endosphere in Bologna were *Proteobacteria* (38.06%), *Actinobacteria* (36.58%), and *Saccharibacteria* (8.31%). In Foggia we had the same three phyla as the most abundant taxa (*Proteobacteria*, 46.73%, *Actinobacteria*, 38.76%, and *Saccharibacteria*, 5.94%).

Our experiment for the first time showed changes of microbiome in the rhizospheric soil and root endophytic microbiome through the Bread Wheat, Wild Emmer Wheat, Domesticated Emmer Wheat, Durum Wheat Landraces, Durum Wheat Cultivars, *T. monococcum* and triticale*.* Furthermore, we have showed that how the fewer tillage and rotation with other crops could

impact the increase of the microbiome communities in wheat which could lead to a better yield and sustainable agriculture.

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Conclusions

Chapter 2

- Soil chemical and structural analysis showed that the values and the variation ranges of soil pH, electrical conductivity (EC), TOC, TN content, total carbonates and bioavailable Cu were within the range of variation typical of vineyards and agricultural soils of Northern Italy.
- There was no difference and benefit in excessive use of Cu for Pignoletto and Alionza grapevine cultivars and nor for the diversity of microorganisms in two vineyards.
- The overall alpha diversity using Shannon index was higher in bacteria rather than fungi, and we have observed the same pattern in both Molinari and Picozzi vineyards with significant differences between the two vineyards.
- In our study, in Molinari vineyard we did not observe bacterial and fungal differences between the two varieties which could imply that the effect of genotype in shaping microbiome might be influenced by other factors
- The beta diversity investigation showed that diversity of bacterial and fungal communities were changing significantly from one vineyard to another one. These differences between Molinari and Picozzi vineyards could be due to several reasons including vary geographical locations and other environmental factors.
- The taxonomical assignments showed that *Proteobacteria* and *Ascomycota* were dominant phyla in vineyard soil and had the highest relative frequencies in bacterial and fungal communities, respectively

Chapter 3

- Minimum tillage management with rotation caused a higher diversity in different wheat species and genotypes in rhizospheric and endophytic samples. In our experiment we have seen a much higher alpha and beta diversity in the field of Bologna which had a minimum tillage management and rotation with *Fabaceae* family in respect to Foggia field which was under conventional management and with no rotation. Hence, it is absolutely crucial to take into consideration the field management for a more sustainable approach.
- In rhizospheric samples, we have seen few but significant differences of prokaryotic diversity between different wheat species in the field of Bologna. However, there was no significant changes of microbiome communities between wheat species in Foggia. The changes of microbiome communities between species could be due to the exudates that changes from one specie to another one. Furthermore, in endophytic samples we have seen more notable changes of microbiome communities between wheat species within each field of Bologna and Foggia but less diverse than the rhizospheric samples.

- In rhizospheric samples within each field of Bologna and Foggia, the microbiome diversities at the plant genetics level have changed significantly.
- The age or developmental stage of the plant was impacting the microbiome communities in rhizospheric samples in Bologna and Foggia. The microbiome diversity using Shannon index in the beginning of ripening stage had a higher diversity in respect to first node stage in Bologna. We have seen a different pattern in rhizospheric samples in Foggia and this could be because of a very different climate that the two locations have and consequently impacting the soil prokaryotic communities. Moreover, the endophytic diversity decreased in the beginning of ripening in respect to first node stage in Bologna. Whilst, the inverse pattern has been seen in endophytic samples in Foggia.
- The taxonomical assignments of rhizospheric samples showed that *Actinobacteria*, *Proteobacteria* and *Planctomycetes* were the most abundant phyla in Bologna and *Actinobacteria*, *Proteobacteria* and *Chloroflexi* were the most dominant phyla in Foggia. Furthermore, the most abundant phyla of endophytic samples in Bologna were *Proteobacteria*, *Actinobacteria*, and *Saccharibacteria*. As in Foggia we had the same three phyla as the most abundant taxa. These taxa were presented in all the wheat species and samples.
- Further taxonomic analysis at the family level, showed that the most frequent rhizospheric bacterial taxa across different wheat species and both fields were *Geodermatophilaceae*, *Microbacteriaceae, Nocardioidaceae, Micromonosporaceae, Propionibacteriaceae, Streptomycetaceae* and *C111* (*Actinobacteria*), *Dolo 23* and *Kouleothrixaceae* (*Chloroflexi*), *Isosphaeraceae* (*Planctomycetes*), *Methylobacteriaceae, Rhodospirillaceae* and *Sphingomonadaceae* (*Proteobacteria*). The most frequent endophytic bacterial family in all wheat species and both fields were *Actinosynnemataceae*, *Microbacteriaceae, Streptomycetaceae, Nocardioidaceae, Micrococcaceae* and *Micromonosporaceae* (*Actinobacteria*), *Rhizobiaceae, Sphingomonadaceae*, *Mitochondria* and *Caulobacteraceae* (*Proteobacteria*).

Appendix 1

Supplementary material – Chapter 2

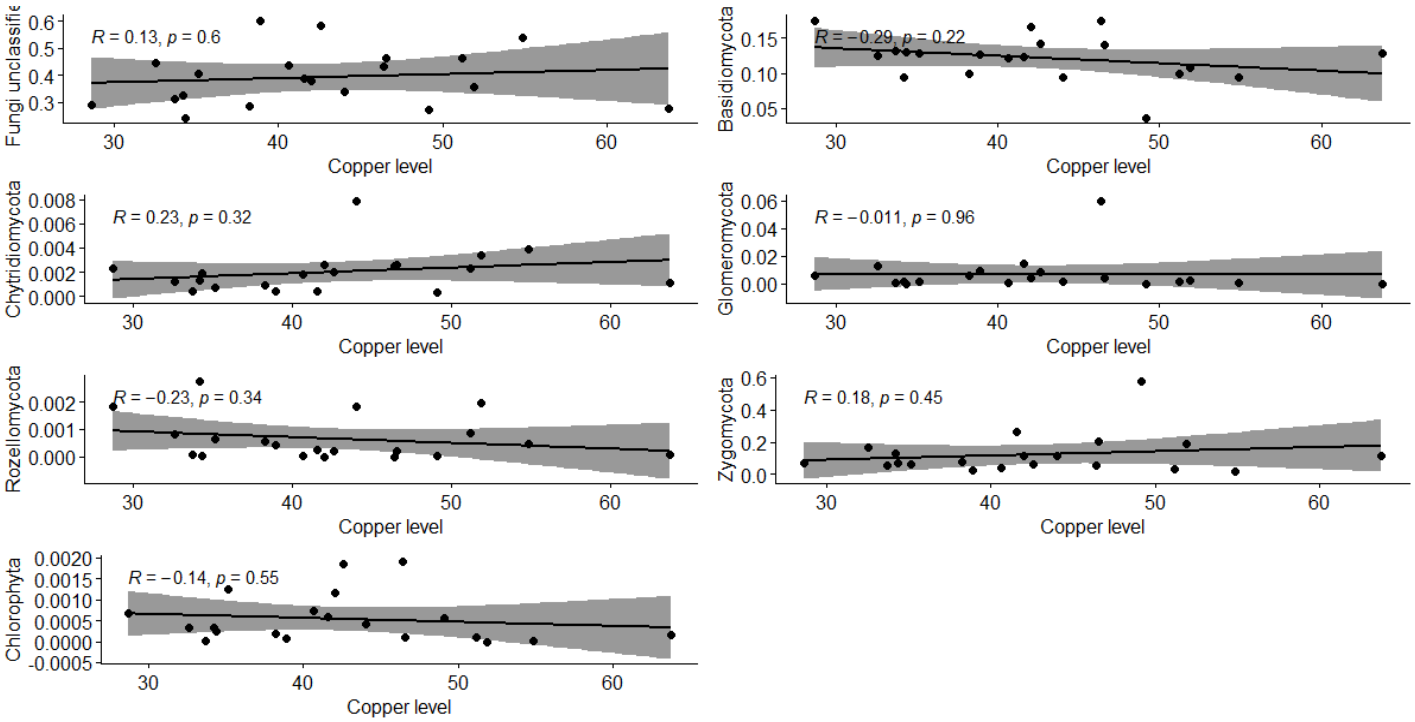

Figure S2.1 Correlation assessment of different fungal phyla with bioavailable Cu in Molinari vineyard. In each plot, the horizontal and vertical axes show the copper level and relative frequency (RF) of the fungi.

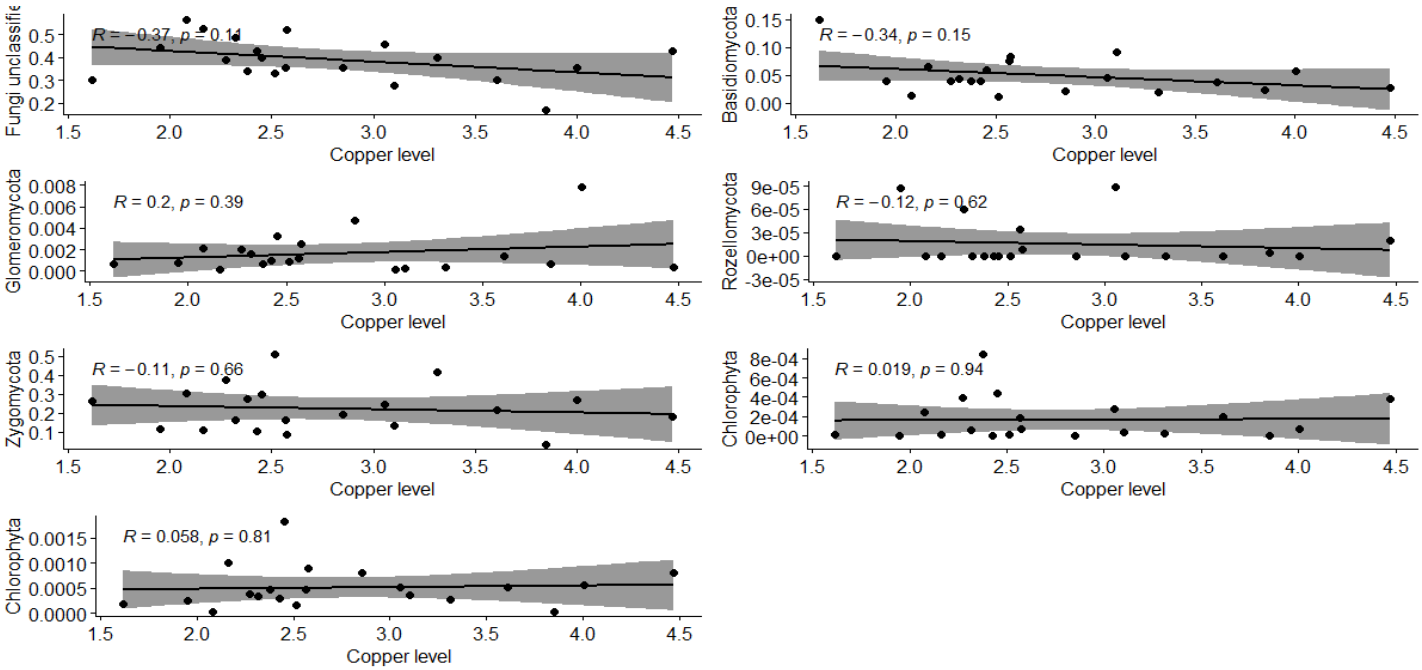

Figure S2.2 Correlation assessment of different fungal phyla with bioavailable Cu in Picozzi vineyard. In each plot, the horizontal and vertical axes show the copper level and relative frequency (RF) of the fungi.

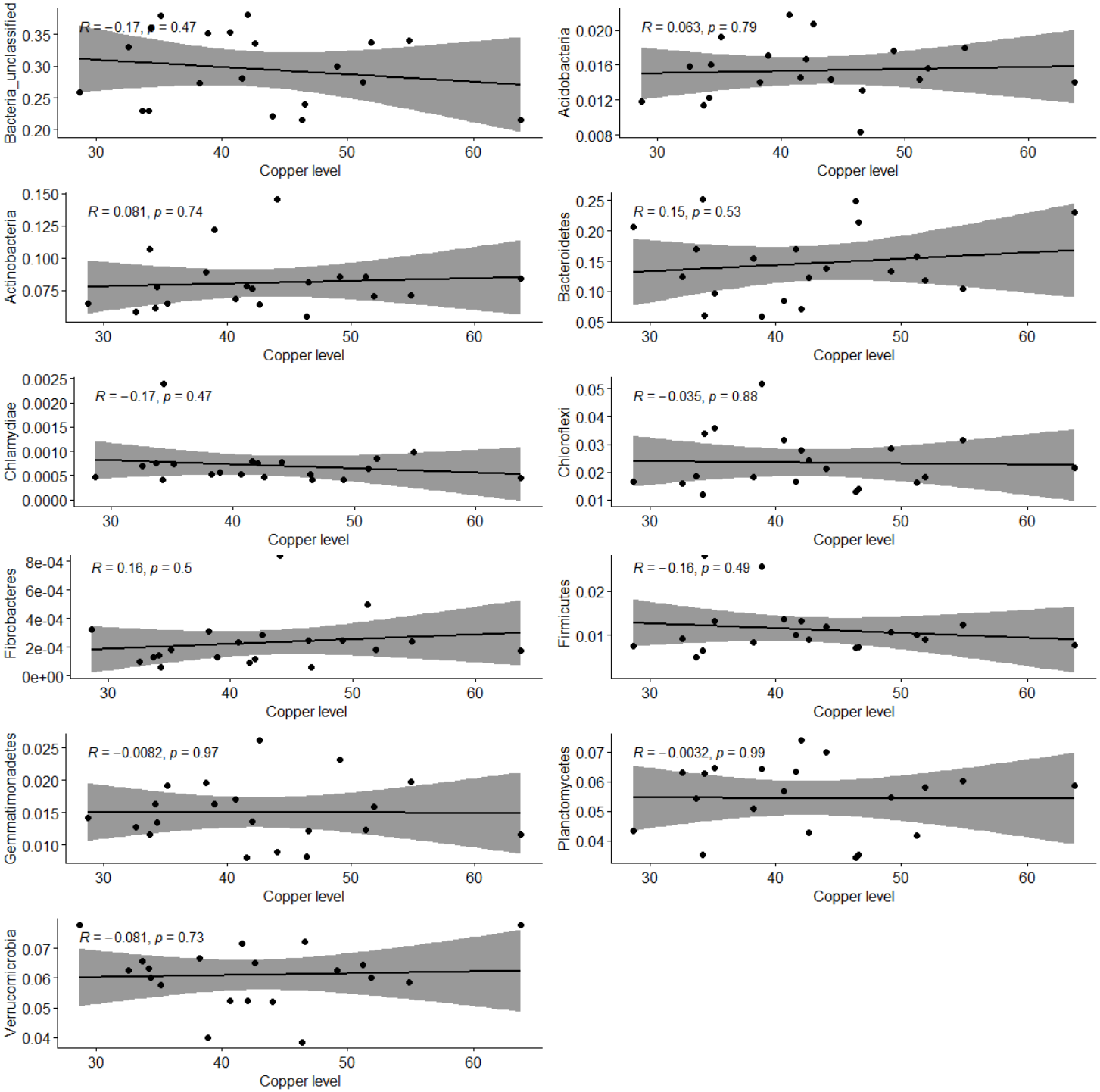

Figure S2.3 Correlation assessment of different bacterial phyla with bioavailable Cu in Molinari vineyard. In each plot, the horizontal and vertical axes show the copper level and relative frequency (RF) of the bacteria.

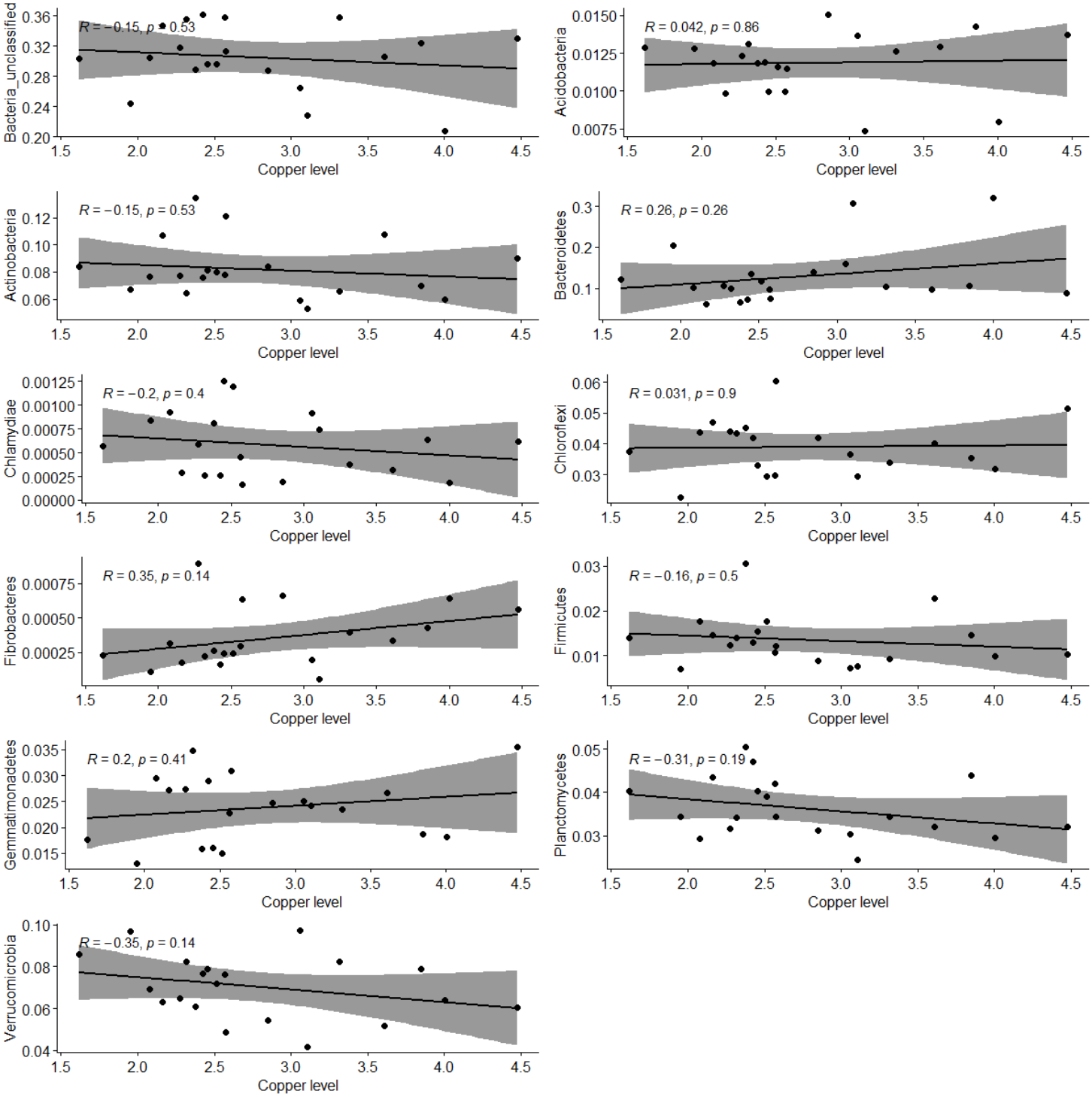

Figure S2.4 Correlation assessment of different bacterial phyla with bioavailable Cu in Picozzi vineyard. In each plot, the horizontal and vertical axes show the copper level and relative frequency (RF) of the bacteria.

Table S2.1 Spearman correlation results for different soil parameters in Molinari and Picozzi vineyards using Shannon, Faith's PD and Evenness indices for bacteria (16S) and fungi (ITS). $^{n.s}$ = not significant

Soil parameters	16S						ITS					
	Molinari (n=28)			Picozzi (n=20)			Molinari (n=28)			Picozzi (n=20)		
	Shannon	Faith's PD	Evenness	Shannon	Faith's PD	Evenness	Shannon	Faith's PD	Evenness	Shannon	Faith's PD	Evenness
Cu	0.15 $^{n.s}$	0.01 $^{n.s}$	0.05 $^{n.s}$	0.25 $^{n.s}$	0.29 $^{n.s}$	0.17 $^{n.s}$	0.09 $^{n.s}$	0.12 $^{n.s}$	-0.08 $^{n.s}$	0.04 $^{n.s}$	0.04 $^{n.s}$	-0.004 $^{n.s}$
Cd	0.23 $^{n.s}$	-0.07 $^{n.s}$	0.004 $^{n.s}$	-0.05 $^{n.s}$	-0.21 $^{n.s}$	-0.06 $^{n.s}$	-0.06 $^{n.s}$	-0.21 $^{n.s}$	-0.08 $^{n.s}$	0.01 $^{n.s}$	-0.36 $^{n.s}$	0.15 $^{n.s}$
Co	0.06 $^{n.s}$	-0.08 $^{n.s}$	0.12 $^{n.s}$	-0.02 $^{n.s}$	0.04 $^{n.s}$	0.16 $^{n.s}$	-0.14 $^{n.s}$	-0.27 $^{n.s}$	-0.06 $^{n.s}$	0.09 $^{n.s}$	0.09 $^{n.s}$	0.04 $^{n.s}$
Cr	0.28 $^{n.s}$	0.23 $^{n.s}$	0.27 $^{n.s}$	-0.06 $^{n.s}$	-0.11 $^{n.s}$	-0.11 $^{n.s}$	-0.13 $^{n.s}$	-0.07 $^{n.s}$	-0.08 $^{n.s}$	-0.23 $^{n.s}$	-0.06 $^{n.s}$	-0.19 $^{n.s}$
Fe	0.01 $^{n.s}$	0.37 $^{n.s}$	0.19 $^{n.s}$	0.16 $^{n.s}$	0.05 $^{n.s}$	0.04 $^{n.s}$	-0.12 $^{n.s}$	0.17 $^{n.s}$	-0.20 $^{n.s}$	0.04 $^{n.s}$	-0.07 $^{n.s}$	0.05 $^{n.s}$
Mn	0.02 $^{n.s}$	-0.24 $^{n.s}$	0.14 $^{n.s}$	-0.03 $^{n.s}$	-0.2 $^{n.s}$	0.07 $^{n.s}$	-0.07 $^{n.s}$	-0.41 $^{n.s}$	0.018 $^{n.s}$	-0.05 $^{n.s}$	-0.28 $^{n.s}$	-0.02 $^{n.s}$
Ni	-0.04 $^{n.s}$	-0.11 $^{n.s}$	0.02 $^{n.s}$	0.01 $^{n.s}$	-0.14 $^{n.s}$	0.21 $^{n.s}$	-0.16 $^{n.s}$	-0.26 $^{n.s}$	-0.12 $^{n.s}$	-0.09 $^{n.s}$	-0.35 $^{n.s}$	-0.02 $^{n.s}$
Pb	-0.002 $^{n.s}$	0.24 $^{n.s}$	-0.13 $^{n.s}$	0.05 $^{n.s}$	-0.1 $^{n.s}$	-0.04 $^{n.s}$	0.02 $^{n.s}$	0.26 $^{n.s}$	-0.07 $^{n.s}$	-0.06 $^{n.s}$	-0.17 $^{n.s}$	-0.04 $^{n.s}$
Zn	0.128 $^{n.s}$	0.37 $^{n.s}$	0.08 $^{n.s}$	0.04 $^{n.s}$	0.18 $^{n.s}$	0.22 $^{n.s}$	-0.07 $^{n.s}$	0.21 $^{n.s}$	-0.08 $^{n.s}$	0.22 $^{n.s}$	0.17 $^{n.s}$	0.16 $^{n.s}$
pH	-0.103 $^{n.s}$	0.04 $^{n.s}$	-0.21 $^{n.s}$	-0.14 $^{n.s}$	-0.07 $^{n.s}$	0.05 $^{n.s}$	-0.01 $^{n.s}$	0.23 $^{n.s}$	-0.18 $^{n.s}$	0.23 $^{n.s}$	0.43 $^{n.s}$	0.12 $^{n.s}$
EC	0.24 $^{n.s}$	0.21 $^{n.s}$	-0.04 $^{n.s}$	0.21 $^{n.s}$	0.31 $^{n.s}$	0.18 $^{n.s}$	0.009 $^{n.s}$	0.19 $^{n.s}$	0.06 $^{n.s}$	0.23 $^{n.s}$	0.03 $^{n.s}$	0.27 $^{n.s}$

Appendix 2

Supplementary material – Chapter 3

Table S3.1 Alpha diversity of rhizospheric soil samples evaluated using pairwise Kruskal-Wallis test for Shannon, Evenness and Faith's PD indices. The P-value adjusted using Benjamini and Hochberg FDR.

Group 1	Group 2	H		
		Shannon	Evenness	Faith_PD
Bologna (n=215)	Foggia (n=216)	268.27^{***}	308.16^{***}	6.83^{**}

$^{n.s}$ = not significant; $^{*}\ p-value < 0.05$; $^{**}\ p-value < 0.01$; $^{***}\ p-value < 0.001$

Table S3.2 Alpha diversity of root endophytic samples evaluated using pairwise Kruskal-Wallis test for Shannon, Evenness and Faith's PD indices. The P-value adjusted using Benjamini and Hochberg FDR.

Group 1	Group 2	H		
		Shannon	Evenness	Faith_PD
Bologna (n=215)	Foggia (n=216)	145.63^{***}	113.22^{***}	$0.004^{n.s}$

$^{n.s}$ = not significant; $^{*}\ p-value < 0.05$; $^{**}\ p-value < 0.01$; $^{***}\ p-value < 0.001$

Table S3.3 Alpha diversity evaluated using pairwise Kruskal-Wallis test for soil samples using Shannon, Evenness and Faith's PD indices in two developmental stages (First node and beginning of ripening) in Bologna and Foggia fields. The P-value adjusted using Benjamini and Hochberg FDR.

Group 1	Group 2	H		
		Shannon	Evenness	Faith_PD
Bologna_First node (n=107)	Bologna_Ripening (n=108)	20.05^{***}	52.29^{***}	15.52^{***}
	Foggia_First node (n=107)	99.58^{***}	144.4^{***}	15.14^{***}
	Foggia_Ripening (n=109)	154.13^{***}	148.45^{***}	14.71^{***}
Bologna_Ripening (n=108)	Foggia_First node (n=107)	126.81^{***}	160.28^{***}	$0.02^{n.s}$
	Foggia_Ripening (n=109)	158.44^{***}	162^{***}	51.29^{***}
Foggia_First node (n=107)	Foggia_Ripening (n=109)	65.03^{***}	9.96^{**}	49.12^{***}

$^{n.s}$ = not significant; $^{*}\ p-value < 0.05$; $^{**}\ p-value < 0.01$; $^{***}\ p-value < 0.001$

Table S3.4 Alpha diversity evaluated using pairwise Kruskal-Wallis test for root endophytic samples using Shannon, Evenness and Faith's PD indices in two developmental stages (First node and beginning of ripening) in Bologna and Foggia fields. The P-value adjusted using Benjamini and Hochberg FDR.

Group 1	Group 2	H		
		Shannon	Evenness	Faith_PD
Bologna_First node (n=107)	Bologna_Ripening (n=108)	12.13^{***}	38.31^{***}	7.10^{*}
	Foggia_First node (n=107)	86.44^{***}	79.02^{***}	$0.21^{n.s}$
	Foggia_Ripening (n=109)	83.73^{***}	96.23^{***}	6.67^{*}
Bologna_Ripening (n=108)	Foggia_First node (n=107)	64.65^{***}	24.11^{***}	5.84^{*}
	Foggia_Ripening (n=109)	56.55^{***}	36.51^{***}	$0.12^{n.s}$
Foggia_First node (n=107)	Foggia_Ripening (n=109)	$2.83^{n.s}$	1.34^{*}	5.82^{*}

$^{n.s}$ = not significant; $^{*}\ p-value < 0.05$; $^{**}\ p-value < 0.01$; $^{***}\ p-value < 0.001$

Table S3.5 Alpha diversity of rhizospheric samples evaluated using pairwise Kruskal-Wallis test for Shannon, Evenness and Faith's PD indices for different species in Bologna and Foggia fields. Wheat accessions of which Bread Wheat (BW), Wild Emmer Wheat (WEW), Domesticated Emmer Wheat (DEW), Durum Wheat Landraces (DWL), Durum Wheat Cultivars (DWC), *T. monococcum* and triticale. The P-value adjusted using Benjamini and Hochberg FDR.

Group 1	Group 2	H		
		Shannon	Evenness	Faith_PD
BW_Bologna (n=18)	BW_Foggia (n=18)	21.63***	26.27***	0.4 n.s
	DEW_Bologna (n=42)	0.21 n.s	5.47*	0.1 n.s
	DEW_Foggia (n=38)	30.35***	36***	1.15 n.s
	DWC_Bologna (n=41)	0.59 n.s	0.6 n.s	0.02 n.s
	DWC_Foggia (n=42)	28.34***	37.18***	0.44 n.s
	DWL_Bologna (n=39)	0.25 n.s	0.01 n.s	0.5 n.s
	DWL_Foggia (n=42)	29.20***	37.18***	2.25 n.s
	Triticale _Bologna (n=16)	0.12 n.s	0.23 n.s	0.2 n.s
	Triticale _Foggia (n=18)	19.62***	25.31***	0.23 n.s
	WEW_Bologna (n=42)	0.94 n.s	1.28 n.s	1.46 n.s
	WEW_Foggia (n=41)	33.39***	36.9***	1.44 n.s
	monococcum_Bologna (n=17)	0.039 n.s	1.26 n.s	0.09 n.s
	monococcum_Foggia (n=17)	24.18***	25.5***	4.75 n.s
BW_Foggia (n=18)	DEW_Bologna (n=42)	29.9***	32.61***	1.46 n.s
	DEW_Foggia (n=38)	0.26 n.s	3.33 n.s	0.11 n.s
	DWC_Bologna (n=41)	36.1***	35.91***	0.98 n.s
	DWC_Foggia (n=42)	0.4 n.s	3.32 n.s	0.01 n.s
	DWL_Bologna (n=39)	29.62***	33.27***	0.02 n.s
	DWL_Foggia (n=42)	0.2 n.s	2.92 n.s	0.73 n.s
	Triticale _Bologna (n=16)	20.43***	24.69***	0.1 n.s
	Triticale _Foggia (n=18)	0.001 n.s	1.16 n.s	0.04 n.s
	WEW_Bologna (n=42)	29.2***	29.73***	0.22 n.s
	WEW_Foggia (n=41)	2.88 n.s	2.39 n.s	0.3 n.s
	monococcum_Bologna (n=17)	21.66***	22.59***	0.48 n.s
	monococcum_Foggia (n=17)	4.46 n.s	0.21 n.s	2.21 n.s
DEW_Bologna (n=42)	DEW_Foggia (n=38)	47.72***	57.2***	3.01 n.s
	DWC_Bologna (n=41)	0.18 n.s	4.2 n.s	0.01 n.s
	DWC_Foggia (n=42)	48.44***	61.13***	2.18 n.s
	DWL_Bologna (n=39)	1.31 n.s	6.27*	1.46 n.s
	DWL_Foggia (n=42)	47.45***	61.7***	4.88 n.s
	Triticale _Bologna (n=16)	0.51 n.s	5.6*	0.53 n.s
	Triticale _Foggia (n=18)	26.31***	32.98***	0.88 n.s
	WEW_Bologna (n=42)	2.71 n.s	0.71 n.s	3.87 n.s
	WEW_Foggia (n=41)	53.76***	56.46***	3.73 n.s
	monococcum_Bologna (n=17)	0.32 n.s	0.57 n.s	0.01 n.s
	monococcum_Foggia (n=17)	32.19***	34.31***	6.3 n.s
DEW_Foggia (n=38)	DWC_Bologna (n=41)	56.64***	58.28***	2.85 n.s
	DWC_Foggia (n=42)	0.2 n.s	0.004 n.s	0.09 n.s
	DWL_Bologna (n=39)	47.02***	56.23***	0.07 n.s
	DWL_Foggia (n=42)	0.002 n.s	0.39 n.s	0.89 n.s
	Triticale _Bologna (n=16)	27.14***	33.16***	0.06 n.s

	Triticale _Foggia (n=18)	0.12 $^{n.s}$	0.02 $^{n.s}$	0.05 $^{n.s}$
	WEW_Bologna (n=42)	46.53***	54.61***	0.04 $^{n.s}$
	WEW_Foggia (n=41)	2.4 $^{n.s}$	0.03 $^{n.s}$	0.15 $^{n.s}$
	monococcum_Bologna (n=17)	29.66***	32.29***	1.19 $^{n.s}$
	monococcum_Foggia (n=17)	4.62$^{n.s}$	1.77 $^{n.s}$	2.18 $^{n.s}$
DWC_Bologna (n=41)	DWC_Foggia (n=42)	57.84***	61.36***	1.67 $^{n.s}$
	DWL_Bologna (n=39)	3.43 $^{n.s}$	0.45 $^{n.s}$	1.21 $^{n.s}$
	DWL_Foggia (n=42)	57.98***	61.5***	5.39 $^{n.s}$
	Triticale _Bologna (n=16)	1.87 $^{n.s}$	0.67 $^{n.s}$	0.73 $^{n.s}$
	Triticale _Foggia (n=18)	32.63***	35.51***	0.98 $^{n.s}$
	WEW_Bologna (n=42)	7.17*	1.06 $^{n.s}$	3.94 $^{n.s}$
	WEW_Foggia (n=41)	60.04***	60.04***	3.35 $^{n.s}$
	monococcum_Bologna (n=17)	1.66 $^{n.s}$	0.99 $^{n.s}$	0.01 $^{n.s}$
	monococcum_Foggia (n=17)	35.04***	35.44***	6.61 $^{n.s}$
DWC_Foggia (n=42)	DWL_Bologna (n=39)	45.55***	59.49***	0.0008 $^{n.s}$
	DWL_Foggia (n=42)	0.09 $^{n.s}$	0.23 $^{n.s}$	0.87 $^{n.s}$
	Triticale _Bologna (n=16)	28.16***	34.17***	0.11 $^{n.s}$
	Triticale _Foggia (n=18)	0.06 $^{n.s}$	0.03 $^{n.s}$	0.002 $^{n.s}$
	WEW_Bologna (n=42)	43.12***	57.69***	0.17 $^{n.s}$
	WEW_Foggia (n=41)	0.7 $^{n.s}$	0.02 $^{n.s}$	0.44 $^{n.s}$
	monococcum_Bologna (n=17)	27.62***	33.92***	1.22 $^{n.s}$
	monococcum_Foggia (n=17)	2.27 $^{n.s}$	2.12 $^{n.s}$	2.91 $^{n.s}$
DWL_Bologna (n=39)	DWL_Foggia (n=42)	45.8***	59.78***	0.98 $^{n.s}$
	Triticale _Bologna (n=16)	0.01 $^{n.s}$	0.06 $^{n.s}$	0.03 $^{n.s}$
	Triticale _Foggia (n=18)	26***	33.27***	0.01 $^{n.s}$
	WEW_Bologna (n=42)	0.69 $^{n.s}$	1.58 $^{n.s}$	0.17 $^{n.s}$
	WEW_Foggia (n=41)	53.16***	56.3***	0.32 $^{n.s}$
	monococcum_Bologna (n=17)	0.03 $^{n.s}$	1.36 $^{n.s}$	0.81 $^{n.s}$
	monococcum_Foggia (n=17)	32.41***	34.27***	2.21 $^{n.s}$
DWL_Foggia (n=42)	Triticale _Bologna (n=16)	27.6***	34.17***	1.28 $^{n.s}$
	Triticale _Foggia (n=18)	0.02 $^{n.s}$	0.02 $^{n.s}$	0.4 $^{n.s}$
	WEW_Bologna (n=42)	44.3***	58.1***	0.49 $^{n.s}$
	WEW_Foggia (n=41)	1.65 $^{n.s}$	0.31 $^{n.s}$	0.11 $^{n.s}$
	monococcum_Bologna (n=17)	28.86***	33.92***	2.42 $^{n.s}$
	monococcum_Foggia (n=17)	3.21 $^{n.s}$	1.66 $^{n.s}$	1.08 $^{n.s}$
Triticale _Bologna (n=16)	Triticale _Foggia (n=18)	17.14***	24.34***	0.08 $^{n.s}$
	WEW_Bologna (n=42)	0.61 $^{n.s}$	0.61 $^{n.s}$	0.24 $^{n.s}$
	WEW_Foggia (n=41)	30.11***	33.93***	0.2 $^{n.s}$
	monococcum_Bologna (n=17)	0.02 $^{n.s}$	1.02 $^{n.s}$	0.25 $^{n.s}$
	monococcum_Foggia (n=17)	22.61***	24***	1.5 $^{n.s}$
Triticale _Foggia (n=18)	WEW_Bologna (n=42)	23.73***	30.44***	0.11 $^{n.s}$
	WEW_Foggia (n=41)	1.01 $^{n.s}$	0.01 $^{n.s}$	0.26 $^{n.s}$

	monococcum_Bologna (n=17)	18.98***	22.59***	0.74 n.s
	monococcum_Foggia (n=17)	2.41 n.s	0.68 n.s	2.01
WEW_Bologna (n=42)	WEW_Foggia (n=41)	53.49***	54.03***	0.01 n.s
	monococcum_Bologna (n=17)	0.67 n.s	0.004 n.s	1.41 n.s
	monococcum_Foggia (n=17)	32***	30.14***	1.88 n.s
WEW_Foggia (n=41)	monococcum_Bologna (n=17)	32.65***	32.46***	1.75 n.s
	monococcum_Foggia (n=17)	0.93 n.s	1.45 n.s	0.87 n.s
monococcum_Bologna (n=17)	monococcum_Foggia (n=17)	23.42***	22.1***	3.92 n.s