Department of Agriculture and Forest Sciences (DAFNE)

in agreement with Enbiotech S.r.l.

PhD Course in

Plant and Animal Science - XXXI Cycle

CHARACTERIZATION OF PLANT-ASSOCIATED MICROORGANISMS WITH BENEFICIAL EFFECTS ON WHEAT GROWTH

(**s.s.d. AGR/16**)

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A.A. 2018/2019

Abstract

The environmental impact of conventional agricultural practices has led to an urgent need of finding alternative solutions for improving agriculture sustainability. In particular, wheat is one of the most important crops in the agro-food sector. Tetraploid wheat production has an important role in Italy where is cultivated mainly in the southern regions, including Sicily. Recently, there was a rising interest in the valorization of local ancient wheat landraces as an important source of genetic and microbial diversity.

To date, the study of the plant microbiota has become particularly important due to its influence on plant physiology and health condition. Indeed, promising results have been obtained so far in the field using manipulated microbiota, allowing the formulation of biofertilizers and biocontrol products that can contribute in lowering the effects of agricultural systems on the environment and human health.

In addition, insect pests are responsible for significant yield losses in many important crops, causing both direct damages to the plant and by transmitting plant viruses. In particular, the cereal-feeding aphids *Rhopalosiphum padi* and *Sitobion avenae* can carry multiple plant viruses and inhibit plant growth on many cereal crops, including wheat. Presently, the principal method adopted to reduce crop damage caused by aphids is insecticide use, which has negative environmental impact and risks development of insecticide resistance.

Plant growth promoting rhizobacteria (PGPR) and several fungal genera are widely studied for their ability of promoting plant growth through several mechanisms, including increasing soil nutrient availability and biocontrol activities. The plant microbiota can also influence plant responses to insect pests. Furthermore, the study of endophytes could be of particular interest for their ability of establishing a mutualistic interaction with the plant. In this framework, the study of beneficial microorganisms is of particular interest as it could allow the development of new bioformulations to improve agriculture sustainability.

Recently, technologies based on spectral data acquisition, including proximal and remote sensing, are acquiring great importance in agriculture for the possibility of measuring parameters associated to plant physiology and health through hyperspectral vegetation indices (HVIs). For this reason, these approaches found broad application in the field of precision agriculture for it does not require destructive sampling and it can provide useful information

that might enhance decision making and a more efficient use of agricultural inputs in specific areas within the field. However, the relationship between hyperspectral data acquired through proximal sensing technology and the composition of plant microbiota or beneficial microbial activities associated to the plant is still poorly understood.

Therefore, the objectives of the present study were: to explore the composition of the microbial community associated to two ancient tetraploid wheat landraces native of the Sicilian territory, Perciasacchi (winter wheat) and Tumminia (spring wheat); to identify wheat-associated HVIs related to plant growth and the presence of beneficial strains or microbial activities. To achieve these goals, a multidisciplinary approach was used involving plate-culturing methods, hyperspectral vegetation index measures through proximal sensing technology, high-throughput sequencing analyses and glasshouse experiments.

These approaches allowed to expand our knowledge about the composition of the endophytic fungal community associated to ancient wheat cultivars. In addition, a group of potential PGPR strains isolated from field-grown plants was selected based on their *in vitro* activities and 16S ribosomal sequence data. The effects of the selected PGPR isolates on plant growth and susceptibility to aphid pests were also evaluated on modern and ancient wheat varieties. Finally, HVIs related to the occurrence of some endophytic fungal genera and to beneficial bacterial activities were also identified.

Aknowledgments

I would like to express my sincere gratitude to Prof. Stefania Masci, Prof. Leonardo Varvaro and Dr. Cosimo Manzo for promoting this joint initiative with University of Tuscia and Enbiotech s.r.l. and for their valuable support, patience and guidance along this path even in the most difficult moments, giving me unconditional trust of great human value.

I will always be grateful to Dr. Ahmed Abdelfattah and Dr. Stefania Gualano for their great dedication, scientific ability and support that guided me throughout this project.

My sincere gratitude to people who have also made key contributions to the scientific success of this thesis: Dr. Sandro Drago for promoting and initiating this research, supporting the activity both in the field and in the laboratory; Dr. Thaer Yaseen for his valuable advice in plant mycology, providing scientific inputs of great relevance to the research; and finally, Dr. Franco Santoro for his precious help in taking the hyperspectral reflectance measures and for his great support in facing the statistical analysis presented in this work.

Sincere thanks to the research group of the laboratory of plant pathology, in particular to Dr. Rocco Caracciolo who guided and helped me at my very first steps with microbiology techniques, for his friendship, endless patience and continuous support during these three years.

I wish to thank Fabio Gallina for giving me access to his field, sown with ancient wheat landraces, where I carried out the first samplings and data collection necessary for the development of this work.

I would like to thank the University of Reggio Calabria for allowing me to work in their laboratories during the study of the endophytic fungal community. In particular, I wish to thank the members of the research group that helped me in this part of my project, including Silvia Scibetta, Maria Giulia Li Destri Nicosia, Saveria Mosca, Antonio Biasi, Sonia Pangallo and David Ruano Rosa.

I am deeply grateful to Dr. Alison Karley for giving me the opportunity of carrying out part of my thesis at The James Hutton Institute, Invergowrie, Dundee (UK). It has been an amazing experience and a pleasure to work under her supervision. I also would like to thank Dr. Carolyn Mitchell, Dr. Susan Mitchell, Dr. Tracy Valentine and Dr. Kirsty Binnie for their precious support and guidance in planning the glasshouse experiments. In addition, I wish to

thank my friends and colleagues met during my Scottish experience for making it so special, including Federica Caradonia, Fulvia Draicchio, Luca Maria Scolari, Gianluca Bretani, Lara Codo, Daniel Leybourne, Marta Barros, Pilar Morera Margarit, Daniel Patko, Ana García-Verdugo Zuil, Amanda Tercero Araque, Nora Quesada, Felipe Pérez Romero, Léa Leroy, Domenico Ronga, Matthias Mimault, Desiré Macheda and in a very special way Magdalena Ślachetka, Amanda Moreno Mellado, Juani and José Manuel Barrera Gavira for their great help when I was under pressure.

Finally, I would like to express my deepest gratitude to my family for their encouragement, sacrifice, strong support and endless love during all my studies I would not be here without them.

Funding

This research has been carried out with the financial support of: Enbiotech S.r.l. (Palermo), the European COST Action FA1405 “Food and Agriculture: Using three-way interactions between plants, microbes and arthropods to enhance crop protection and production” and the Italian Ministry of Education, University and Research with the grant “Innovazione di prodotto e di processo nelle filiere dei pro-dotti da forno e dolciari” (PON03PE_00090_01).

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Chapter 1

Introduction, objectives, experimental scheme

Introduction

Intensive agronomic practices are raising environmental concerns due to the extensive use of fertilizers and chemicals for plant nutrition and protection against pathogens and pests with adverse effects in terms of nutritional quality of the crops and environmental pollution (Ram *et al.*, 2014; Pankhurst *et al.*, 1996). Consequently, the need of developing lower impact alternatives to reduce chemical applications led to the study of plant-associated microbial communities, known as plant microbiota, for their ability of influencing plant development and tolerance to stresses (Berendsen *et al.*, 2012; Berg, 2009). The possibility of manipulating the plant microbiota to improve agricultural sustainability brought to several studies investigating the composition and interactions of the plant microbiota associated to many important crops (Abdelfattah, 2016; Bulgarelli *et al.*, 2013), including wheat (Germida and Siciliano, 2001). Recently, the interest in ancient landraces is raising for they represent an important source of microbial and genetic diversity for improving modern crops (Nazco *et al.*, 2012). In addition, recent technologies have been used to study microbial communities through culture-independent approaches using high throughput sequencing (HTS). Other technologies based on measures of hyperspectral reflectance, such as proximal sensing, have found broad application in agriculture, mainly in the field of plant pathology, allowing to measure spectral vegetation indices linked to plant health and physiology in order to improve the use of resources in the agricultural systems (Alnaasan, 2015; Rinaldi *et al.*, 2015). However, this approach might be suitable for evaluating the effects of the plant microbial community on plant health and physiology as well as to improve sampling schemes in order to collect representative samples in a given population (Hamada *et al.*, 2014). In this work, a multidisciplinary approach including culture-dependent and independent approaches, along with proximal sensing, was applied in order to provide useful insights on the composition of the microbial community associated to ancient wheat landraces and to evaluate the presence of beneficial strains suitable for the improvement of plant growth in modern wheat varieties.

The plant microbiota

Plants are associated with microbial communities, known as plant microbiota, that can colonize plant tissues and influence health and growth of their host through several mechanisms, including nutrient uptake and plant tolerance of biotic and abiotic stresses. Plant associated microbial communities are usually grouped in three classes based on their interaction with the host, including pathogens, mutualists and commensals (Berlec, 2012). Scientific studies have focused mainly on pathogens in order to prevent crop losses caused by plant diseases, providing the main part of available information about plant-microbe interactions (Berlec, 2012). However, the study of rhizobia associations of leguminous plants and nitrogen-fixing bacteria drove the attention of the scientific community on mutualistic interactions (Masson-Boivin et al., 2009). Commensals have not caught immediate attention as they were considered "neutral actors" within the microbiota, providing neither harm nor benefit to the host (Berlec, 2012). However, recent studies showed that the composition of commensals is regulated by the plant (Gottel et al., 2011) and the function of the plant microbiota contributes to the host's phenotype, leading to a wider and more relevant attention to the study of plant-microbe interactions (Vorholt et al., 2017).

Host colonization

Plant-associated microorganisms can colonize several plant surfaces and tissues that represent different ecological niches and are usually grouped in three main compartments: phyllosphere, rhizosphere and endosphere (Müller et al., 2016).

- *Phyllosphere*

The phyllosphere is the aerial part of the plant, including stem and leaves, with a lower availability of nutrients and a more dynamic and complex environment, in terms of microbial structure compared with the rhizosphere (Yang et al., 2001). Indeed, microbes colonizing aerial plant tissues are influenced by leaf structures (veins, hairs and stomata) as well as by exposure to abiotic factors such as radiation, wind, temperature, precipitation and moisture (Turner et al., 2013).

- *Rhizosphere and plant- growth-promoting rhizobacteria*

The rhizosphere is the region of soil influenced by plant roots with the release of exudates, mucilage and sloughed cells. These compounds attract and shape the surrounding microbial

community. According to the studies, the microbial distribution of this area among cultivars is similar at the phylum level, whereas differences can be evidenced at the species and strain levels (Turner et al., 2013). The rhizosphere has been deeply studied, because it is where plant nutrient uptake takes place, and for the presence of plant growth-promoting rhizobacteria (PGPR). PGPRs have been highly studied in the last years for their beneficial effects on plant growth through direct and indirect mechanisms. Direct plant growth-promoting (PGP) activities include production of growth-regulating hormones, such as indoleacetic acid (IAA), and increase of soil nutrient availability, with consequent enhancement of plant nutrients uptake performed by nitrogen-fixers, inorganic phosphorous (Pi) and potassium (K)-solubilizing microorganisms (Kumar *et al.*, 2015). Indirect beneficial activities include siderophores and hydrogen cyanide (HCN) production that are involved in biocontrol mechanisms and induction of plant systemic defense (Ahmad and Khan, 2008).

In the last years, there was a raise in the studies focused on PGPRs and several species of bacteria (such as *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia)* have been reported to enhance plant growth (Singh *et al.*, 2015; Kumar *et al.*, 2012; Mrkovacki and Milic, 2001; Dashti *et al.*, 1998; Freitas and Germida, 1990). Among them, species belonging to *Bacillus* and fluorescent *Pseudomonas* have been widely studied for the wide range of activities performed by these groups of bacteria, such as nitrogen fixation, production of plant growth regulators and siderophores, and solubilization of inorganic phosphate (Saxena and Tilak, 1998; Saharan and Nehra, 2011). In particular, fluorescent pseudomonads were considered of particular interest for field applications, as they grow easily in plate culture, show ability of quickly colonizing plant roots, compete with other microorganisms, can adapt to environmental stresses and induce systemic acquired resistance (SAR) in the plant (Saharan and Nehra, 2011; Van Peer *et al.*, 1991). Furthermore, production of siderophores, such as pyoverdine or pseudobactins, is one of the strength features of fluorescent *Pseudomonas* strains. These compounds are produced in iron-limiting conditions to chelate ferric iron and transport it in the bacterial cell (Neilands, 1981). Furthermore, siderophores production by fluorescent *Pseudomonas* has been linked to biocontrol activities against plant pathogens ,probably due to a better competitiveness in iron intake (Becker and Cook, 1988).

- *Endosphere*

The endosphere environment includes the internal tissues of the plant which is colonized by endophytic microorganisms. Endophytes are generally defined as microorganisms that

colonize plant internal tissues without causing apparent disease symptoms to their host (Petrini, 1991). In addition, the interactions between endophytes and their host may include phytopathogenic as well as mutualistic traits depending on several factors, including environmental conditions, host defenses and developmental stage of the host and the endophyte (Schulz and Boyle, 2005). Some endophytic strains can be isolated in plate culture from surface-sterilized plant tissues (Tan and Zou, 2001).

Bacterial endophytes have been classified as "obligate", when the interaction with a living plant is needed to complete their lifecycle, (Döbereiner *et al.*, 1993) or "facultative", that can be selected by the plant and establish a mutualistic interaction (Kamnev *et al.*, 2005). The latter group includes a wide range of species most of them belonging to *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Reinhold-Hurek and Hurek, 2011; Rosenblueth and Martínez-Romero, 2006). Nowadays, the important role played by bacterial endophytes for their host has been assessed by numerous evidences of the beneficial activities performed by these bacteria, including the enhancement of nutrient intake by the plant (e.g. nitrogen fixation), biocontrol and bioremediation activities (Santoyo *et al.*, 2016; Subramanian and Smith, 2015).

Among fungal endophytes, mycorrhizal fungi have been deeply studied (Simon *et al.*, 1993). However, non-mycorrhizal endophytic fungi and yeasts are also able to colonize below- and above-ground plant tissues and some of them can establish beneficial interactions with the plant by producing growth hormones (e.g. indoleacetic acid) (Nassar *et al.*, 2005) and through enhancement of drought stress tolerance, as demonstrated in wheat by Hubbard and co-workers (2014). In addition, coexistence of pathogenic and beneficial strains in many crops, including grasses, has also been reported even though their interactions are still poorly understood. Recently, reduced disease symptoms of *Puccinia triticina,* the agent of leaf rust in wheat plants by fungal endophytes, has been reported (Dingle and McGee, 2003).

Source of inoculum and determinants of composition of plant microbiota

The plant microbiota may vary based on the plant species or cultivars, plant compartments, geographic location, developmental stage and plant health conditions (Figure 1). Moreover, several studies showed highly variable results regarding the role played from these drivers in shaping the microbial communities (Berg et al., 2016; Müller et al., 2016).

Microbial composition of the plant microbiota is determined mainly by environmental exchange and soil represents the main source of microorganisms for the rhizosphere (Müller

et al., 2016). However, vertical transmission from seeds can occur with endophytes (Bulgarelli et al., 2013; Rosenberg et al., 2009).

Differences in species richness was observed in microbial communities of bulk soil, rhizosphere, root surface and the endosphere (Müller et al., 2016). These observations suggest a strong action played by the host plant in recruiting microorganisms from the soil. Indeed, shifts in the community composition in response to environmental changes and plant development have been reported observing a decrease in microbial diversity during plant growth, probably regulated by the release of plant metabolites and by the plant immune system (Chaparro et al., 2014; Turner et al., 2013).

Finally, microbial competition for space and nutrient within the plant ecological niches cannot be excluded from the determinants of the composition of the plant microbiota (Müller et al., 2016). In addition, abiotic and biotic environmental factors have been shown to affect the composition of the plant microbiota, including agricultural practices, climate conditions or severe infections (Berlec, 2012).

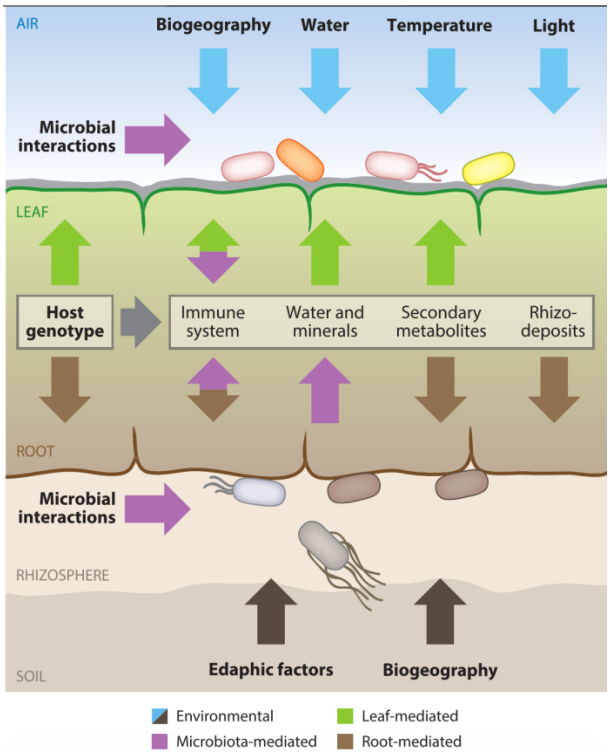

Figure 1. Factors and interactions determining the composition of the plant microbiota (Müller et al., 2016)**.**

Impact of plant domestication on the plant microbiota

Considering the active role of the host genotype in recruiting microbial species from soil, the loss of genes due to plant domestication might have influenced the composition of the plant microbiota associated with modern crops (Bulgarelli et al., 2013; Wissuwa et al., 2009). Indeed, the selection performed on wild relatives throughout the centuries allowed to improve their productivity (enhance seed size, loss of seed dispersal), seed quality (decrease of bitter substances) and growth patterns (Gross and Olsen, 2010). However, genetic selection along with changes in agricultural management practices, contributed in reducing plant genetic diversity with an increasing need of external inputs, such as the application of fertilizers and pesticides (Pérez-Jaramillo et al., 2016; Matson et al., 1997). It is reasonable to imagine that all these changes had consequences on the composition of the plant microbiota and its interactions with the host. Indeed, it has been demonstrated a negative impact of long-term nitrogen fertilization on mutualistic rhizobia, with reduced benefit to the host (Weese et al., 2015).

Studies conducted on the rhizospheric community of several crops showed that ancient landraces were associated to a higher microbial diversity compared to modern cultivars (Szoboszlay et al., 2015; Germida and Siciliano, 2001). In addition, wild relatives were often used as resources of genetic traits to improve resistance of modern cultivars to abiotic and biotic stresses (Pérez-Jaramillo et al., 2016). Therefore, traits associated with microbial-recruitment might be of high interest for breeding programs, as well as studies of the plant microbiota of ancient landraces might lead to the identification of beneficial strains to be transferred to modern cultivars in order to improve the environmental impact of agricultural practices (Berlec, 2012).

Approaches to study the plant microbiota

Plant microbiota has traditionally been studied focusing on culturable bacterial species, using several growth media, and their interaction with the plant. Although this culture-dependent approach provides valuable data and a pure culture is required for detailed genetic and physiological studies, it cannot consider the whole microbial diversity in an environment, which includes unculturable strains also (Turner et al., 2013).

Nowadays, culture-independent techniques have been introduced for the study of the plant microbiota. Usually, these techniques are based on DNA amplification using polymerase-

chain reaction (PCR) of the ubiquitous 16S ribosomal RNA (rRNA) gene, whereas for the study of eukaryotic microbes, such as fungi, the hypervariable internally transcribed spacer is often used (Tedersoo et al., 2015). In particular, the development of high-throughput sequencing technologies have allowed the acquisition of thousands to millions of sequences in an environmental sample, including plant tissues, revealing the structure and the relative abundance of different phylogenetic groups within the microbial community (MacLean et al., 2009). However, even this approach has its limitations due to the complexity of the plant microbiota, requiring large sequencing coverage and the need of replicated experimental designs with consequent high financial costs (Bulgarelli et al., 2013; Knight et al., 2012).

The combination of culture-dependent and independent techniques might benefit from both approaches and compensate their limitations for the study of the plant microbiota (Schlaeppi and Bulgarelli, 2015).

The most common approach relays on culture-dependent screenings to identify potential PGPRs to be further analyzed; however, there are ongoing attempts to screen beneficial isolates directly in the field (Finkel et al., 2017). Usually, studies aimed to evaluate the role of plant-growth promoting microorganisms are carried out in controlled conditions. On one end, this approach excludes some biotic and abiotic drivers shaping the plant microbiota (Hamonts et al., 2018); on the other hand, a simplified environment is helpful to evaluate the effects of single strains. In addition, despite the complexity of the variables involved in the study of the plant microbiota, there is a growing evidence of the existence of a "core microbiota" including heritable microbial taxa, related to plant health and performance (Hamonts et al., 2018). This concept, along with the expansion of microbial collections with beneficial strains, might have an important impact on the development of microbial combinations, known as "synthetic microbiota", with advantages in the study of plant-microbe interactions and for field applications (Schlaeppi and Bulgarelli, 2015).

In this work, culture-dependent screening methods have been used in order to isolate and screen potential PGPRs from plant tissues of two ancient Sicilian wheat landraces (Kumar *et al.*, 2012; Ahmad *et al.*, 2008), Perciasacchi and Tumminia. Considering the deep interaction established by endophytes with the plant host and the numerous beneficial roles reported by some endophytic species, both culture-dependent and culture-independent approaches were used to study the endophytic microbial community associated to the two ancient wheat landraces mentioned above. Finally, pre-screened PGPRs were further tested in glasshouse experiments in order to assess their role on both ancient and modern wheat varieties.

Wheat

Phenology

Wheat is a cereal grain belonging to the family of Poaceae. Globally, the most widespread species of wheat is the hexaploid *Triticum aestivum*, which is also known as bread wheat. However, *Triticum spelta* and *Triticum durum* are also broadly cultivated. The latter is particularly important in the Mediterranean countries, including Italy, where tetraploid wheat is used for production of pasta and some types of bread (Nazco *et al.,* 2012; Raffo *et al.,* 2003).

Wheat can grow as winter or spring cereal based on their sowing times. Winter wheat is usually sown in autumn, it requires a vernalization time during winter and it grows from spring to summer; whereas spring wheat is sown in spring and grows until autumn (Bowden *et al.,* 2008).

Wheat growth consists of three main phases (Bowden *et al.,* 2008). The first phase includes germination, leaf development, and tillering, when growth of roots, leaves, and tillers take place. Tillers are secondary plants having their own culm and several leaves and they are connected to the main plant by internodes in the root system. After leaf development, winter wheat requires a vernalization stage of at least six to nine weeks, with a temperature from 0–5°C during which plant growth is interrupted. The second phase is the reproductive cycle including stem elongation, booting, heading and flowering. After the heading phase, senescence occurs starting from the ear and expanding upwards and downwards (Zadoks *et al.,* 1974). Finally, in the third growth phase plant physiological maturity and grain development occur. Wheat phenology can be measured according to several scales, such as the BBCH scale of Meier and colleagues (2009) and the Feekes scale which was introduced by Zadoks and co-workers (1974).

Ancient wheat landraces in Sicily

Recently, the valorization of local ancient wheat landraces is acquiring a great importance. Indeed, modern wheat varieties are the result of thousands of years of domestication process, using several breeding strategies in order to improve grain yield and quality, as well as to facilitate the harvest (Dubcovsky and Dvorak, 2007; Motzo and Giunta, 2007). However, the loss of genes caused by the domestication process is one of the major problems of genetic

breeding, therefore ancient wheat landraces are becoming an important source of genetic diversity providing resistance and nutritional traits (Ellis et al., 2014; Nazco et al., 2012; Uauy et al., 2006).

Due to the active role of the host genotype in recruiting plant-associated microbial diversity (Sapkota *et al.,* 2015) and considering the adaptation of ancient landraces to drought and the low nutrients requirements, as well as their importance on the genetic point of view, native ancient landraces might be an important source of microbial diversity for improving the modern ones (Doebley *et al.,* 2006; Eyre-Walker *et al.,* 1998).

Tetraploid wheat is the primary cereal crop grown in the southern regions of the Italian peninsula and the islands, including Sicily (Motzo and Giunta, 2007). In Sicily, flours obtained from two ancient tetraploid wheat landraces, Perciasacchi (winter wheat; *Triticum turgidum* subs. *turanicum*) and Tumminia (spring wheat; *Triticum durum*), also known as "Timilia" (Perrino and Hammer, 1983), are still used in local traditional products and are considered particularly valuable for their peculiar organoleptic properties (Vita *et al.,* 2016). Therefore, in the present work, the possibility of finding beneficial microorganisms associated to these two landraces has been investigated, with particular focus on root-associated and endophytic plant-growth promoting rhizobacteria (PGPR) and endophytic fungi.

Proximal sensing

Technologies based on spectral data acquisition, such as proximal and remote sensing, found broad application on the field of precision agriculture for the possibility of measuring parameters associated to plant physiology and health (Fourty *et al.*, 1996) without destructive sampling procedures. These approaches can also provide information about plants located in specific areas within the field enhancing the decision making and allowing a more efficient use of agricultural inputs (Mulla, 2013).

Leaf optical properties

The study of the leaf optical properties has been developed in the last thirty years. These properties are based on the physics principle that any material exposed to different wavelengths of electromagnetic energy (EM) can reflect, absorb and transmit a light radiation in different ways. Therefore, different materials can be uniquely characterized based on their specific *spectral signature*. Usually, the main part of the diagnostic absorbance properties of the green vegetation fall within the optical range of the EM spectrum (Kokaly *et al*., 2009).

The leaf optical properties represent the percentage of incident radiation that is reflected (reflectance), absorbed (absorbance) and transmitted (transmittance) by the leaf surface (Figure 2). When these characteristics are measured using a spectroradiometer (hyperspectral data) the spectra obtained are usually between 350 and 2500 nm of wavelength and are characterized by different trends based on the plant species, cultural and nutritional conditions, and possibly biotic and abiotic stresses of the plants.

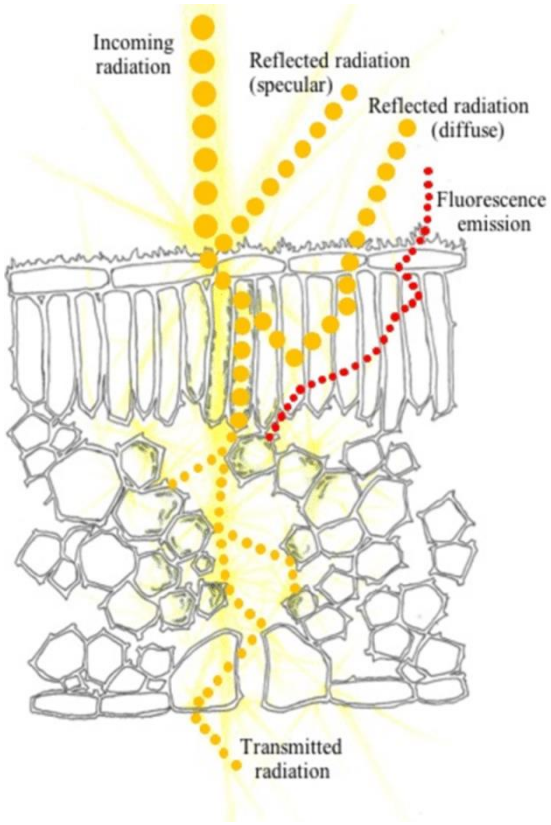

Figure 2. Partitioning of the incoming light radiation as reflected, transmitted, or absorbed energy (Olascoaga, 2018).

Hyperspectral reflectance

Several studies carried out on a large number of herbaceous, horticultural and arboreal species in the recent years showed a very close correlation between reflectance percentages and leaf characteristics in different agricultural conditions.

In particular, narrow-band spectral (or hyperspectral) reflectance has improved the estimates of vegetation parameters and their plant traits compared to traditional multispectral data that provided limited information with few and typically wide spectral bands (Goetz, 2009).

Spectral reflectance data can provide useful information on the plant biophysical and biochemical parameters. To date, hyperspectral data have been used to recover non-pigment biochemical parameters such as nitrogen, phosphorus, lignin/cellulose and water content as well as pigment-associated parameters, including carotenoids, anthocyanins and chlorophylls (Casas *et al*., 2014; Axelsson *et al*., 2013; Wang *et al*., 2012; Qu *et al*., 2008; Daughtry *et al*., 2000).

These measures can also provide information on biophysical characteristics (morpho-structural parameters) of the plant, including plant and leaf biomass in a given area, leaf area,

stem diameter and average height of an arboreal plant (Guerschman *et al*., 2009; Darvishzadeh, 2008).

Vegetation indices

Reflectance data with hundreds of narrow bands (hyperspectral) allowed to determine plant specific bioindicators through the definition of "unique" hyperspectral vegetation indices (HVIs) using two, three or more of the available bands.

Several studies conducted on spectral signatures of vegetation led to the definition of some indices, derived from ratios between reflectance at certain wavelengths, particularly effective in describing certain physiological and/or stress conditions of the plant. Furthermore, indices obtained from reflectance values allow measurements on both small and large scale (Peñuelas and Filella, 1998).

The ratio of reflectance measured at 698 nanometers (nm) and the one measured at 760 nm is closely related to chlorophyll concentration (Moran *et al*., 2000), whereas the ratios of reflectance measured respectively at 695 and 420 nm and at 695 and 760 nm are closely related to several stress conditions due to herbicides application, pathogen infections, ozone damage, foliar senescence and tissue dehydration (Carter, 1994).

Using these information there are several well-known vegetation indices reported in literature including the Simple Ratio Index (SRI), which is defined as the ratio between the bands of near-infrared (NIR) and visible red (R); the Normalized Difference Vegetation Index [NDVI = (NIR - R) / (NIR + R)], related to the amount of green biomass (per unit area) and to the density of absorbed photosynthetic photon flow (PPFD) (Peñuelas and Filella, 1998). Moreover, the Photochemical Reflectance Index [PRI= (R531-R570)/(R531 + R570] is strongly correlated to the de-oxidation state of the xanthophyll cycle and, along with the Normalized Phaeophytinization Index [NPQI= (R415-R435)/(R415+R435)], provides indications on photosynthetic efficiency and thermal extinction of the photosystem II (Peñuelas and Filella, 1998).

Furthermore, there are several indices closely related to other leaf characteristics, including anthocyanin content, nitrogen nutrition (Reyniers *et al*., 2006), carotenoids/chlorophyll ratios (Peñuelas *et al*., 1995) and water content (Peñuelas and Inoue, 1999).

Table I shows the main biophysical and biochemical vegetative indices reported in literature for the detection of plant diseases and variations in plant physiology.

Table I. List of some hyperspectral vegetation indices cited in literature and related to plant physiology.

Acronym	HVI	Formula	Reference
PRI	**Photochemical Reflectance Index**	$(R_{531} - R_{570}) / (R_{531} + R_{570})$	Gamon *et al.*, 1992
NDVI	**Normalized Difference Vegetation Index**	$(R_{800} - R_{670}) / (R_{800} + R_{670})$	Rouse Jr *et al.*, 1974
MCARI	**Modified Chlorophyll (a and b) Absorption in Reflectance Index**	$[(R_{701} - R_{670}) -0.29 * (R_{701} - R_{550})] * (R_{701} / R_{670})$	Daughtry *et al.*, 2000
ChlNDI	**Chlorophyll Normalized Difference Index**	$(R_{750} - R_{705}) / (R_{750} + R_{705})$	Gitelson and Merzlyak, 1994
WI	**Water Index**	$(R_{900}) / (R_{970})$	Penuelas *et al.*, 1995
PSRI	**Plant Senescence Reflectance Index**	$(R_{680} - R_{500}) / R_{750}$	Mishra and Arora, 2016
SIPI	**Structure Insensitive Pigment Index**	$(R_{800} - R_{445}) / (R_{800} + R_{680})$	Gitelson and Merzlyak, 1994
REP	**Red Edge Position**	$R_{700} + 40*[((R_{670} - R_{780}) / 2) - R_{700}] / (R_{740} - R_{700})$	Merzlyak *et al.*, 1999
YI	**Yellow Index**	$- (R_{580} + R_{668} - 2*(R_{624})) / (0.044\text{^}2)$	Adams *et al.*, 1999
Vi_{opt}	**Optimal Vegetation Index**	$(1.45) \times ((R_{800})^2 + 1)/(R_{670} + 0.45)$	Reyniers *et al.*, 2006
PSNDa	**Pigment Specific Normalized Difference**	$(R_{800} - R_{680})/(R_{800}+R_{680})$	Blackburn, 1998
PSNDb	**Pigment Specific Normalized Difference**	$(R_{800} - R_{635})/(R_{800}+R_{635})$	
PSNDcar	**Pigment Specific Normalized Difference**	$(R_{800}-R_{470})/(R_{800}+R_{470})$	

mSR	**Modified Simple Ratio**	$\left(\frac{R_{800}}{R_{670}} - 1\right) / \sqrt{\left(\frac{R_{800}}{R_{670}} + 1\right)}$	Chen, 1996
ARI	**Anthocyanin Reflectance Index**	$(1/R_{550}) - (1/R_{700})$	Gitelson *et al.*, 2001
BIG2	**Blue/Green Index**	R_{450} / R_{550}	Zarco-Tejada *et al.*, 2005
DD	**Double Difference Index**	$(R_{749} - R_{720}) - (R_{701} + R_{672})$	le Maire *et al.*, 2004
NPQI	**Normalized Phaeophytinization Index**	$\frac{R_{415} - R_{435}}{R_{415} + R_{435}}$	Barnes *et al.*, 1992
mCAI	**Modified Chlorophyll Absorption Integral**	$\frac{(R_{545} - R_{752})}{2}(752 - 545) - \left(\sum_{R_{545}}^{R_{752}} R \times 1.423\right)$	Laudien *et al.*, 2003
RVSI	**Red Edge Vegetation Stress Index**	$\frac{R_{714} + R_{752}}{2 - R_{733}}$	Naidu *et al.*, 2009
Lic	**Lichtenthaler Indices**	R_{440}/R_{690}	Lichtenthaler, 1996

This technology could offer interesting applications for investigating plant-microbe relationships (Hamada *et al*., 2014) by identifying HVIs related to wheat physiological growth and with the presence of beneficial microorganisms. Therefore, proximal sensing was used in this work to identify HVIs associated to wheat health and correlated with plant height measures. In addition, correlations between HVIs, the composition of the fungal entophytic community and plant growth promoting activities performed by plant-associated bacteria were also investigated.

Objectives

Based on the promising results obtained so far in the use of beneficial microorganisms to enhance wheat growth and health along with the growing need of finding more environmentally friendly solutions to manage our agricultural systems, the present study has been carried out in order to reach the following objectives:

- application of proximal sensing for planning a more precise sampling scheme using plant vegetation indices (VIs) and evaluation of the correlation between HVIs and plant height;
- investigation of the composition and interactions of the endophytic fungal community associated to two ancient wheat landraces, Perciasacchi (winter wheat) and Tumminia (spring wheat), looking for the presence of potential beneficial species;
- isolation and screening of potential PGPR strains belonging to the two mentioned ancient wheat landraces in order to select active isolates to be tested *in vivo*;
- evaluation of the effects of potential PGPR strains on plant growth and tolerance to insect pests in both ancient and modern wheat varieties.

In order to reach these aims, a multidisciplinary approach has been used including proximal sensing, culture-dependent methods, molecular biology techniques, high throughput sequencing analyses, statistical analyses and glasshouse experiments.

Experimental sites and sampling schemes

The tetraploid wheat landraces studied in this work were grown in two fields located in the Madonie area of the Sicilian inland. The first, termed here "Field 1" (37.7813410 N, 14.2852990 E), was located at 850 m above sea level and previously used for legume cultures while "Field 2" (37.740245 N, 14.239368 E) was located at 800 m above sea level and was uncultivated for almost five years.

Samples of bulk soil were collected from both fields in the first week of November 2015, before sowing. Each field was divided into four sections and 10 soil samples were randomly collected into each section at 20 cm under soil surface. Soil samples were transported to the laboratory, dried into clean cardboard boxes for 24 h and sifted before proceeding with bacterial isolation (Figure 3).

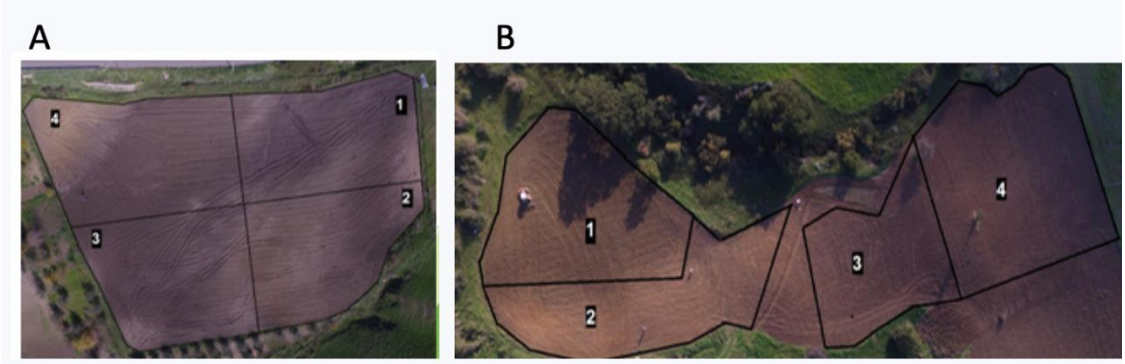

Figure 3. Sampling scheme of bulk soil in field 1 (A) and field 2 (B).

Afterwards, one half of both sites was sown with the winter wheat Perciasacchi in the second week of November 2015, whereas the other half was sown with the spring wheat Tumminia in the first week of February 2016. The seeds and the fields have not undergone to any treatment neither before nor after sowing in order to recover local microorganisms. In addition, based on the orography of each field 46 sampling points were placed in field 1 and 43 in field 2.

The geographic position of each point has been registered using the Global Positioning System (GPS; Figure 4).

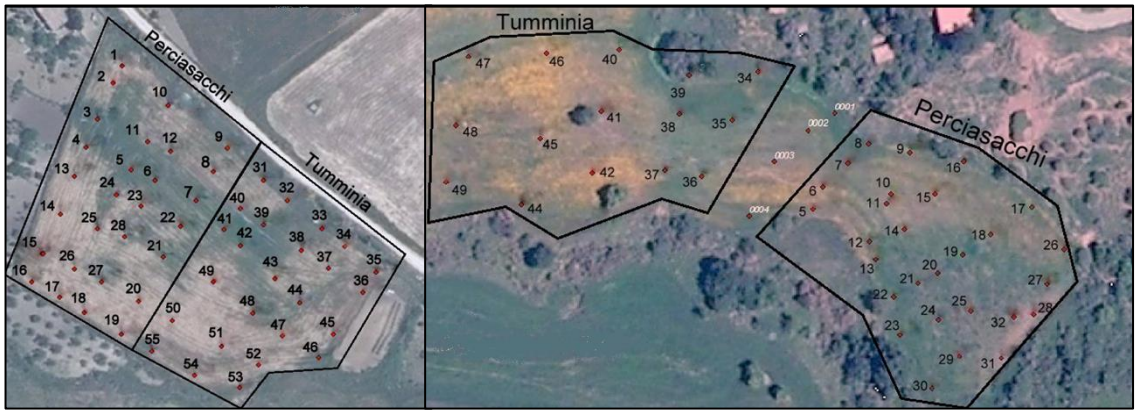

Figure 4. Distribution of sampling points in the two fields under study. Field 1 (left) and field 2 (right) were sowed with both wheat landraces under study: Tumminia and Perciasacchi.

Sampling of Perciasacchi roots and both varieties stems was performed during stem elongation (March 2016) and heading (May 2016) phases, respectively. Ten plants, including the radical system, were collected in a radius of 50 cm from each sampling point. Wheat roots and stems were transferred to the lab and processed within 72 h as described in the next chapters.

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Chapter 2

Evaluation of plant performance using morphologic measures and proximal sensing approach

Abstract

The study of plant-associated microbial diversity is raising a great interest for the possibility of finding useful microorganisms to be used in the field in order to improve plant growth and reducing the application of chemical inputs. So far, the most used method for isolating new plant-beneficial strains is destructive sampling using randomized schemes. In addition, isolation and identification of beneficial microbial strains requires time-consuming and expensive laboratory procedures. Recently, proximal sensing technology is covering an important role in the field of precision agriculture allowing to calculate hyperspectral vegetation indices (HVIs) related to chemical and physiological parameters associated to important crops, including wheat. However, the relationships between HVIs and the plant-associated microbial community is still poorly studied. Moreover, ancient tetraploid wheat varieties are considered an important source of genetic and microbial diversity. Considering the important role played by plant-microbe interactions for plant health and physiology, proximal sensing could provide useful information about the presence of beneficial microorganisms or microbe-associated activities that might be used for planning more efficient sampling schemes, targeted to specific areas of the field. Therefore, in this study the correlation between seven HVIs related to important plant parameters and plant growth, expressed in terms of plant height, is reported by studying two ancient tetraploid wheat landraces.

Introduction

Tetraploid wheat is particularly important in Italy for its flour is used for the production of pasta and some types of bread. Recently, a growing interest in ancient varieties of wheat of southern Italy (source of genetic and microbial biodiversity) has attracted a scientific curiosity about the possibility of finding beneficial microorganisms associated with these plants that could be transferred to modern varieties to improve the growth and health of plants (Doebley *et al*., 2006; Eyre-Walker *et al*., 1998). The possibility of detecting the presence of microbial activities with benefic effects on plant growth directly in the field would allow to focus on certain areas of the field for sampling and isolation of useful microorganisms. Moreover, more precise recommendations might be given on the application of fertilizers and their quantity (Pilbeam, 2015), with a positive impact on the environment and on the profitability of farmers.

To date, there are no conventional methods or monitoring procedures for determining the presence of such microorganisms in space and time. Indeed, the methods used so far to detect beneficial fungi and bacteria are based on destructive massive plant sampling, laborious, expensive and time-consuming isolation and laboratory screening procedures of environmental microorganisms associated with plant tissues (see Chapters 3, 4 and 5). Consequently, the development of new field investigation criteria would bring to targeted and more efficient sampling schemes. The use of optical sensors for spectral reflectance measurement, through the spectroscopic technique of proximal sensing (see Chapter 1), led to new methods and opportunities for the estimation of vegetation chemical and physical properties in a rapid, non-destructive and relatively cheap way, allowing a better management of agricultural inputs of crops (Shi *et al*., 2015; Hatfield *et al*., 2008).

In this context, proximal sensing could be used to obtain information on wheat nutrition and health conditions through the study of spectral reflectance (Gualano, 2017; Rinaldi *et al*., 2015). Moreover, this approach could provide a useful contribution to the study of the relationships between the plant "spectral signatures" and the microbial community associated with wheat (Hamada *et al*., 2014).

Recently, several optical bio-indicators have been identified (spectral bands and hyperspectral vegetation indices) to compare different wheat growth stages and yield parameters in the presence of differentiated fertilization using a common data set within the farm (Diacono *et al.*, 2014; Li and Gao, 2010; Xavier *et al.*, 2006). These parameters have also been correlated

to morphological measurements such as plant height (Xavier *et al*., 2006). However, to date, the use of spectral reflectance to assess the growth status of the plant in ancient landraces of tetraploid wheat in relation to the beneficial microorganisms associated with them has not yet been explored. Consequently, one of the aims of this thesis was to use hyperspectral vegetation indices (HVIs) available in literature and correlated with morphological growth parameters (plant height) of two ancient tetraploid wheat varieties native of Sicily (the winter wheat Perciasacchi and the spring wheat Tumminia), in order to establish an efficient sampling criterion allowing to include most of the microbial biodiversity associated with these landraces grown in two different locations. The resulting sampling scheme was used for the study of the fungal endophytic communities associated to the wheat landraces under study (see Chapter 3) and the isolation and screening of bacteria with beneficial effects on plant growth from bulk-soil and plant tissues (see Chapter 4).

Finally, univariate statistical analyses were carried out to establish the degree of correlation between the HVIs detected on wheat plants and the microbial communities and beneficial activities detected using the sampling scheme based on proximal sensing data.

Materials and methods

Field location, sampling and plant preparation

The sampling of wheat plants was carried out according to the sampling scheme previously described in Chapter 1, section "Experimental sites and sampling schemes".

On the basis of the established experimental design, 10-15 wheat plants were randomly eradicated in each field within a radius of approximately 50 cm from each geolocalized sampling area (Figure 5). The aerial part of the plants was cut using scissors, one leaf was randomly selected from each stem and cleaned with absorbent paper. Spectral signatures were acquired immediately after cutting in a controlled environment.

The harvested wheat roots and stems were transported to the laboratory and used for the isolation of bacteria to be screened for plant growth promotion activities, as described in Chapter 4.

Figure 5. Example of sampling area (code M05).

Leaf spectral measurements

Spectral reflectance measurements were carried out on leaves of the two landraces under study at three different time points (expressed in Day of the Year - DoY) corresponding to three wheat growth stages (see "Wheat" section of Chapter 1 for more details):

- Stem elongation (March 2016 - DoY 73);
- Heading (May 2016 - DoY 131);
- Ripening/maturation (June 2016 - DoY 161).

Hyperspectral data were collected using an acquisition system (Figure 6) consisting of: a FieldSpec®3 spectroradiometer (Analytical Spectral Device, Boulder, USA), which measures in the near infrared-visible (VIS-NIR: spectral range 350-1000 nm) and in the medium infrared (SWIR: spectral range 1000-1830 nm) with spectral sampling intervals of 1.4 and 2 nm, respectively; and a light contact probe and a leaf clip (plant probe: Analytical Spectral

Device, USA) connected to the spectroradiometer through a fiber optic cable and a portable PC for displaying and saving the spectral signatures.

Figure 6. Hyperspectral acquisition system. *On the left side are shown the tools used for hyperspectral data acquisition; on the right is shown an example of wheat leaves sample.*

In order to acquire high quality spectra, the spectroradiometer was turned on and left heating for about 30 minutes and the optical fiber cable was immobilized using sticky tape to prevent noises caused by its movement. Hyperspectral data were acquired by placing the leaf clip holder of 10 mm diameter in the middle of the leaf, beside the midrib to collect uniform measurements. In addition, all collected reflectance spectra (interpolated to 1 nm) were displayed and saved using the RS^{3}TM Spectral Acquisition Software (ASD, Boulder, USA). Moreover, for each measurement the device performed an automatic procedure of calibration and light optimization using a white reference (Spectralon®, Labsphere, USA) which guaranteed homogeneous and normalized spectra.

Plants morphometric measurements

Plant height is an important morphological and developmental indicator of the overall growth of the plant which is largely predictive of biomass and final grain yield (Wang *et al*., 2018). Theoretically, it is defined as the minimum distance between the upper limit (the highest point) of the main photosynthetic tissues (excluding inflorescences) and the soil level (Pérez-Harguindeguy *et al*., 2016). Therefore, morphometric measurements of plant height were taken in the field within each sampling point without eradicating the plants and following the same timing of reflectance measurements. Measurements of 12-15 randomly chosen wheat stems, within a radius of 50 cm from each geolocalized point, were performed using a rigid

meter. Plant height data were registered on an Excel file (Microsoft) using a tablet and then analyzed using statistical methods.

Pre-processing: optimization of hyperspectral data

Raw spectral signatures acquired through the RS3TM software (DoY 73, 131 and 161) in the proprietary "asd.ref" format were pre-processed using the ViewSpec Pro 6.0.10 software (ASD Inc.). Smoothing and instrumental noise removal and spectrum alignment were performed in order to obtain spectral signatures with 1430 available wavelengths in the visible and infrared regions at 10 nm intervals each. All spectral signatures were exported in ASCII text format and used for generating datasets.
In addition, the reflectance measurements were performed "by contact" with the leaves, therefore the “scaling” of the spectra to brightness changes was not necessary. Moreover, the light emitted by the halogen lamp inside the plant-probe interface produces a constant spectrum (similar to the solar one) and all the reflectance measurements carried out were carefully calibrated through "light optimization" and "white reference" procedures.

Selection of a group of Vegetation Indices

Chemical variations within and between plant species have a strong influence on the ecological interactions between plants and their abiotic and biotic environment. These variations are based on genetic differences (Pichersky and Gang, 2000), phenological stage of plants (Hartmann and Zimmer, 1986), climate, presence of herbivores or microbial communities in the soil (Joosten *et al*., 2009; Shiojiri and Karban, 2008; Macel *et al*., 2004). The latter are often unevenly distributed in the field (Van der Putten *et al*., 2001; Masters and Brown, 1997) and this heterogeneity might produce spatial variations in chemical components of plants belonging to the same variety (Bartelheimer *et al*., 2010). These differences can be found on all classes of primary compounds: nutritional, including nitrogen (N) and chlorophyll (Chl), and defensive, such as alkaloids and glucosinolates (Baldwin *et al*., 1999; Kliebenstein *et al*., 2001).

So far, the use of spectral models provided by the reflected light of plant leaves for detecting interspecific and/or intraspecific chemical differences between plant species in relation with soil biotic conditions has not been demonstrated. However, starting from the 1430 spectral reflectance values available, the calculation of the most representative HVIs reported in the

literature and listed in Table I (see Chapter 1), that resulted to be related to the macro classes of primary compounds mentioned above, has been carried out.

A total of 17 hyperspectral indices were selected and calculated, four of which were used to estimate the concentration of chlorophyll pigments (*Pigment Specified Simple Ratio Chlorophyll*, **PSSRa** and *Pigment Specified Simple Ratio Chlorophyll b*, **PSSRb**), carotenoids (*Pigment Specified Simple Ratio Carotenoids*, **PSSRc**) and anthocyanins (*Anthocyanin Reflectance Index*, **ARI**); four for the mean chlorophyll content and physiological/defenses/nutritional status (*Red Edge Position*, **REP**; *Chlorophyll Normalized Difference Index*, **ChlNDI**; *Yellowness index*, **YI** and *Chlorophyll indices*, (**Index_SPAD**); two for photosynthetic and thermal efficiency of the photosystem II (*Photochemical Reflectance Index* (**PRI**) and *Normalized Phaeophytinization Index* (**NPQI**). In addition, the *Water Index* (**WI**), which indicates changes in water status; the *Plant Senescence Reflectance Index* (**PSRI**), used to detect the onset and degree of senescence of plants; the *Healthy index*, (**HI**) and the *Blue/Green Index* (**BIG1**), related to abiotic/biotic stress conditions (defense)**, t**he *Normalized Difference Vegetation Index* (**NDVI**) and the *Optimal Vegetation Index* (**Vi_{opt}**) related to plant physiological growth and nutritional condition.

The work of Rizzuti *et al.* (2018) showed that leaf maturity involves the visible region and region of the spectrum ranged between 800 and 1.300 nm (plateau near and medium infrared). In particular, the range between 800 e 900 nm of the spectral signature belonging to leaves of different plant species is characterized by negative solpes in young leaves and positive slopes in older or senescent leaves. Based on these observations, a new index, "Slope", has been tested which is defined by the following equation:

$$Slope = \frac{(R_{900} - R_{800})}{(900 - 800)}$$

Figure 7. Equation defining the "Slope" index. *R_{800} and R_{900} are the reflectances at wavelengths 800 and 900 nm respectively.*

Therefore, this index has been considered as plant growth indicator based on the maturation stage of the leaves for the two ancient wheat landraces under study.

Statistical analyses

Data on the average of plant height in the three phenological growth stages (stem elongation, heading and maturation) were analyzed through the descriptive parameters of the main statistical mean values (Valid N, Mean, Minimum, Maximum, Variance, Skewness and Kurtosis) according to the variety groups (Perciasacchi, Tumminia), field (Field 1, Field 2) and day of the year DoY (73, 131 and 161).

Moreover, due to the heterogeneity in data distributions, the non-parametric Spearman correlation was applied to analyze the interactions between the hyperspectral vegetation indices and the average of plant height. The "relationship ratios" and "functional structure" between the average of plant height (growth indicator) and the selected hyperspectral indices (predictors) were evaluated. Therefore, the most correlated hyperspectral indices were further studied for their potential relationship with the spatial diversity of the microbial communities (endophytic fungi) and plant-growth promoting activities (wheat-associated bacteria) to be investigated as described in the next chapters. In addition, the Regression analysis was used to study the relationships between the predictors.

The validity of the statistical model was tested through a study on the residuals resulted from the difference between the observed and the expected values based on the regression model. All the analyses were carried out using Statistica v.8 run in a Windows environment (StatSoftinc.).

Results

Hyperspectral signature acquisition

The summary of the results obtained from the acquisition of hyperspectral signatures from wheat leaves belonging to Perciasacchi and Tumminia grown in the two fields under study is reported in Table II.

Table II. Summary of the hyperspectral signatures of wheat leaves. *The total count of the hyperspectral signatures acquired from the leaves of the two landraces under study (Perciasacchi and Tumminia) in the two fields at the three Days of Year (DoY) is reported.*

		Spectral signature [count]		
Landrace	**DoY**	***Field 1***	***Field 2***	***Total.***
Perciasacchi	73	204	319	523
Tumminia	73	-	-	-
Perciasacchi	131	403	343	746
Tumminia	131	152	-	152
Perciasacchi	161	160	129	289
Tumminia	161	200	149	349

Moreover, as Tumminia is a spring wheat landrace, it was sown in February 2016, therefore measures at DoY73 could not be taken due to the small dimensions of the leaves. In addition, Tumminia plants grown in field 1 were less developed at DoY 131 compared to the same variety grown in field 2, therefore hyperspectral measures could be taken only on plants grown in field 2.

An example of the variability detected in the spectral signatures acquired from the leaves of Perciasacchi plants grown in field 1 at the three DoYs is shown in Figure 8.

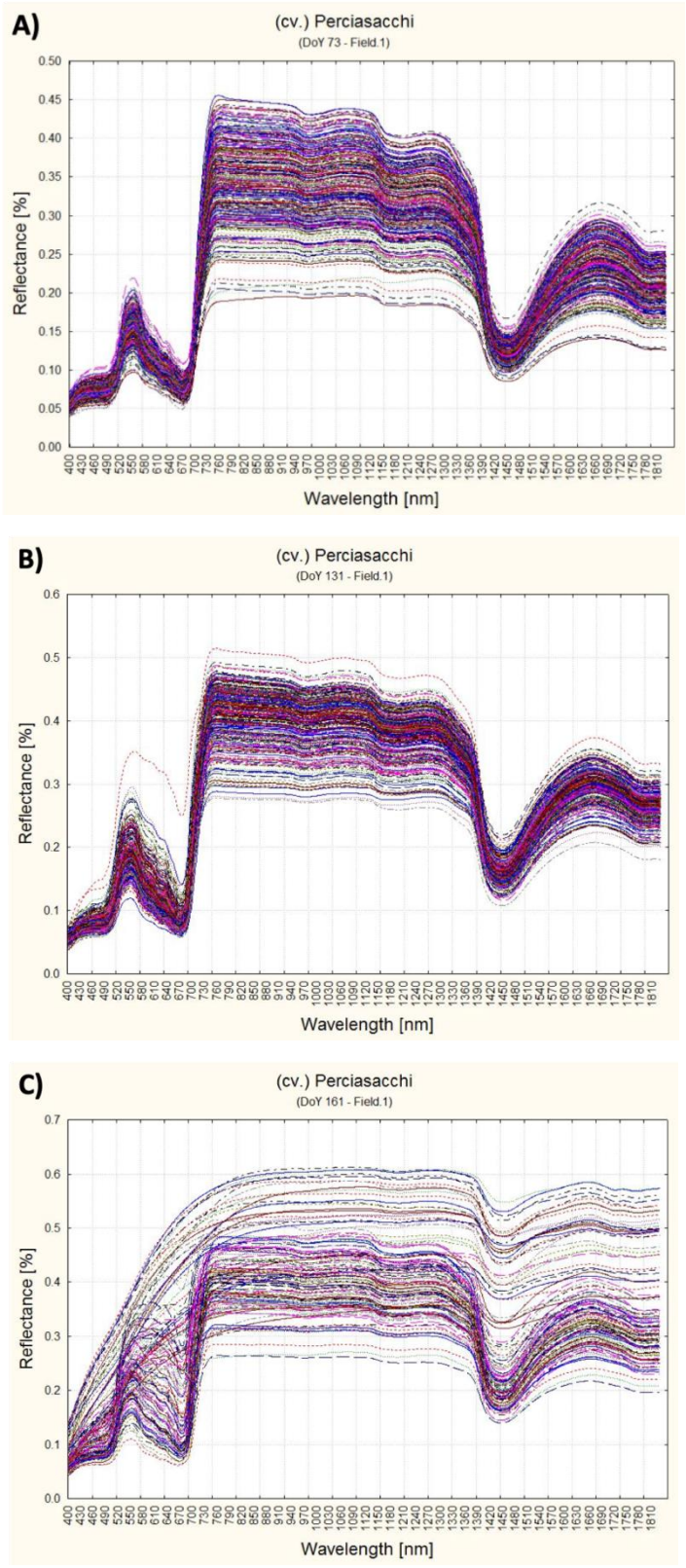

Figure 8. Hyperspectral signatures acquired from Perciasacchi plants grown in field 1 at DoYs 73 (A), 131 (B) and 161 (C). *Variability of the spectral signatures can be observed in the visible (400-700 nm) and the infrared region (300 – 1830 nm).*

Variability of the spectral signatures detected in Perciasacchi plants at DoYs 73, 131 and 161 changes based on the growth stage. Indeed, at DoYs 73 and 131 (Figure 8A and B) most of this variability is found in the visible region (VIS: 400- 700 nm), especially the blue (~450-495 nm) and red (~650-680 nm) and in the infrared region of shortwave (SWIR: 1300-1830 nm).

In addition, observing the reflectance curves in the green region (~520-610 nm), at DoY 131 Perciasacchi plants showed higher variability of reflectance values compared with DoY 73 (Figure 8A).

The relationship between the hyperspectral curves and wheat physiological condition can be observed also in the near infrared region (NIR: 800-1300 nm) which is related to the internal structure of the leaf. Indeed, in younger leaves, at DoY 73, the slope has a decreasing trend, whereas at DoY 131 it is almost horizontally oriented and at DoY 161 most of the curves show an increasing trend. Similar observations can be done by looking at the water absorption regions in the NIR (970 nm) and SWIR (1180 and 1450 nm).

Morphometric measures

In Table III are reported the average values of the main descriptive statistical parameters of plant height (APH) measured in each geolocalized sampling area at the three DoYs (73, 131 and 161), in the two fields and in the two ancient wheat landraces.

Table III. Summary of the morphometric measures acquired from wheat plants. LR: *landrace;* ***P:*** *Perciasacchi;* ***T:*** *Tumminia.*

			Dependent Variable: *Average plant_height*						
DoY	**LR**	**Field**	**Valid N**	**Mean**	**Minimum**	**Maximum**	**Variance**	**Skewness**	**Kurtosis**
73	*P*	*1*	28	43.9	26.6	55.6	66.17	-0.71	-0.04
		2	28	36.8	20.4	57.7	92.13	0.45	-0.28
	T	*1*	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-
131	*P*	*1*	14	107.9	62.9	136.4	407.33	-0.97	0.65
		2	28	86.6	48.8	115.9	481.80	-0.19	-1.48
	T	*1*	-	-	-	-	-	-	-
		2	14	47.5	31.0	65.7	130.69	0.25	-1.36
161	*P*	*1*	28	104.2	76.0	132.1	165.78	0.07	0.35
		2	26	89.8	53.4	114.3	254.53	-0.62	-0.38
	T	*1*	20	67.2	53.2	79.8	63.98	-0.26	-1.16
		2	14	60.4	36.7	80.5	138.93	-0.22	0.02

Considering that Tumminia plants (spring wheat) were sown in February 2016, at DoY 73 the plants did not reach the stem elongation phase, therefore plant height measures were taken at DoY 131. Unfortunately, due to non-optimal weather conditions, Tumminia plants grown in field 1 could not be measured at DoY 131.

Moreover, skewness and kurtosis values reported in Table III are very close to zero indicating that all height measures taken were normally distributed. The trends of the "Mean" values reported in Table III are shown in Figure 9, in the same experimental conditions.

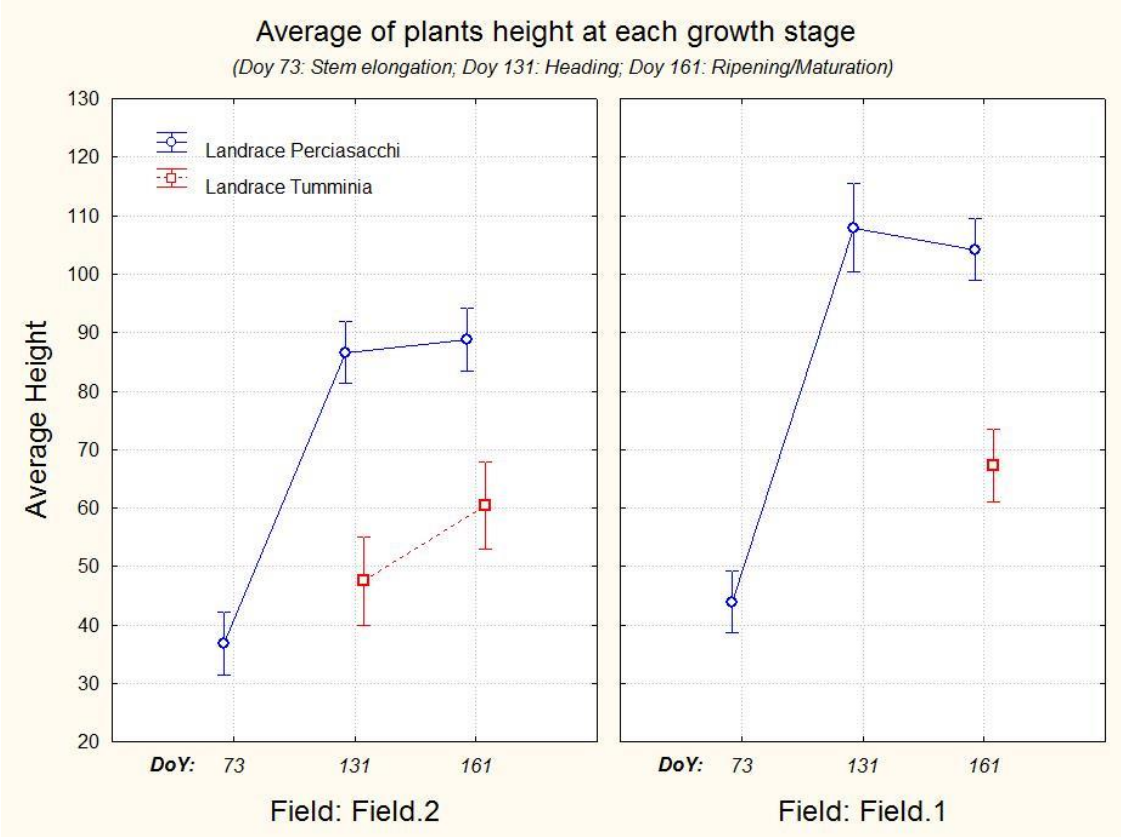

Figure 9. Trends of the mean values of plant height of Perciasacchi and Tumminia at the three Days of year (DoYs) in the two fields.

From the trends of APH, shown in Figure 9, significant changes in plant growth can be observed in Perciasacchi plants in the two fields from DoY 73 and DoY 131, whereas no APH variation is observed between DoY 131 and DoY 161 in the two fields. Therefore, data regarding this landrace at DoY 161 were excluded from further statistical analyses.

Regarding Tumminia plants, despite the lack of data at DoYs 73 and 131 in field 1, data collected from DoYs 131 and 161 in field 2 allowed to evaluate the growth trend of this landrace in this period of time.

Wheat growth-related indices of hyperspectral vegetation

Hyperspectral reflectance data acquired from wheat leaves (10 – 15 for each sampling area) were averaged in order to obtain a mean of the spectral signatures representative of the sampling area (1 m^2). The average of spectral reflectances in the different wavelengths have been used to calculate 17 previously selected HVIs (see paragraph "Hyperspectral reflectance" of Chapter 1) through the mathematical relationships reported in Table I and Figure 7.

An exploratory statistical analysis was carried out on the calculated HVI values to verify their asymmetry and kurtosis (indicators of data normality conditions).

Table IV shows the overall average results obtained for both varieties and for the entire growth phase (DoYs 73, 131 and 161).

Table IV. Summary of average values of hyperspectral vegetation indices (HVIs) acquired at the three Days of Year.

HVI	Mean	Variance	Standard Error	Skewness	Kurtosis
Slope	**-0.029**	**0.000**	**0.001**	**4.341**	**38.552**
PRI_{570}	-0.007	0.000	0.001	0.331	0.073
Vi_{opt}	3.142	0.006	0.006	0.297	-0.663
$PSSR_a$	4.572	0.141	0.030	-0.306	-0.491
$PSSR_b$	3.803	0.188	0.034	-0.339	-0.125
$PSSR_{car}$	4.940	0.210	0.036	0.326	0.792
NDVI	0.654	0.001	0.002	-0.627	-0.005
ChlNDI	0.319	0.002	0.004	-0.328	-0.059
ARI	**-0.099**	**0.032**	**0.014**	**3.716**	**30.494**
WI	**1.024**	**0.000**	**0.000**	**-2.500**	**14.151**
BGI2	0.471	0.002	0.003	-0.171	-0.130
PSRI	**-0.002**	**0.000**	**0.001**	**0.944**	**5.103**
REP	**639.371**	**60.772**	**0.616**	**-1.309**	**2.198**
NPQI	-0.067	0.000	0.001	-0.318	0.768
YI	**-1.727**	**2.904**	**0.135**	**1.913**	**4.685**
IndexSPAD	0.624	0.002	0.004	-0.588	0.070
HI (Healthy Index)	-0.070	0.000	0.002	-0.032	-0.065

The table shows important deviations from the assumptions of normality for some of the selected indices (highlighted in bold). In particular, the Slope, ARI, WI, REP and YI showed

the highest deviations, whereas all the others fulfilled the conditions of normality (values in absolute terms close to zero).

Based on the deviation of data from normal distribution, showed in the previous table, Spearman's non-parametric correlation index was used to establish possible relations between the spectral indices and the average of plant height (Table V).

Table V. Results of Spearman's correlation performed on the average of plant height and the HVIs.

Variable & Index:	**Spearman R**	**p-level**
Average_Height&Slope	-0.733	0.00000
Average_Height& PRI570	**-0.152**	**0.06767**
Average_Height&Vi_{opt}	0.705	0.00000
Average_Height&PSSRa	0.561	0.00000
Average_Height&PSSRb	0.421	0.00000
Average_Height&PSSRcar	0.642	0.00000
Average_Height& NDVI	0.563	0.00000
Average_Height&ChlNDI	0.387	0.00000
Average_Height& ARI	**0.077**	**0.35652**
Average_Height& WI	**0.144**	**0.08216**
Average_Height& BGI2	-0.218	0.00818
Average_Height& PSRI	**-0.010**	**0.90447**
Average_Height& REP	0.349	0.00002
Average_Height& NPQI	-0.277	0.00070
Average_Height& YI	**0.081**	**0.33183**
Average_Height&Index_SPAD	0.468	0.00000
Average_Height&HI_Healthy_Index	-0.315	0.00011

Consistent and significant correlations were observed between the average height measured on both ancient wheat landraces with the hyperspectral indices related to the nutritional conditions Slope, Vi_{opt} and NDVI. The group of hyperspectral indices associated with chlorophyll a and b (PSSRa, PSSRb), carotenoids (PSSRcar) and mean chlorophyll/nutritional content (IndexSPAD) showed slightly lower significant positive correlations. The remaining indices showed very low or not significant correlations.

Finally, a simple regression analysis was conducted in order to highlight the relationship between the most correlated indices (Slope, Vi_{opt}, PSSRcar and PSSRa) with the average of plant height (Table VI).

Table VI. Summary of the Regression analysis performed between the average of plant height and the most correlated spectral vegetation indices.

Dependent Variable:	**R**	**R^2**	**Adjusted R^2**	**F**	**p-level**	**Std.Err. of estimate**
Slope	0.77	0.59	0.59	208.35	0.00000	0.007
Variables currently in the Equation:	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			-0.0094	0.0015	-6.37	0.00000
AverageHeight	0.7701	0.0533	-0.0003	0.0000	-14.43	0.00000
Model: Slope = -0.0094 - 0.0003*Average Height						
Dependent Variable:	**R**	**R^2**	**Adjusted R^2**	**F**	**p-level**	**Std.Err. of estimate**
Vi_{opt}	0.78	0.61	0.60	220.74	0.00000	0.045
Variables currently in the Equation:	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			3.0035	0.0094	320.50	0.00000
Average_Height	0.7790	0.0524	0.0021	0.0001	14.86	0.00000
Model: Vi_{opt} = 3.0035 + 0.0021*Average Height						
Dependent Variable:	**R**	**R^2**	**Adjusted R^2**	**F**	**p-level**	**Std.Err. of estimate**
PSSRa	0.53	0.28	0.27	54.52	0.00000	0.318
Variables currently in the Equation:	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			4.0854	0.0665	61.45	0.00000
Average_Height	0.5254	0.0712	0.0073	0.0010	7.38	0.00000
Model: PSSRa = 4.0854 + 0.0073*Average Height						
Dependent Variable:	**R**	**R^2**	**Adjusted R^2**	**F**	**p-level**	**Std.Err. of estimate**
PSSRcar	0.67	0.45	0.44	116.02	0.00000	0.299
Variables currently in the Equation:	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			4.2491	0.0625	67.94	0.00000
Average_Height	0.6693	0.0621	0.0100	0.0009	10.77	0.00000
Model: PSSRcar = 4.2491 + 0.010*Average Height						

In all four analyses performed the partial regression coefficient B of Average Height was different from zero with a highly significant Student t value. Moreover, the F values were very high and significant especially for the growth-related indices Vi_{opt} and Slope. R^2 values were also interesting, explaining 61% and 59% of the overall dispersion of the average height

compared to the other two pigment-related values (45% of PSSRcar and 28% of PSSRa). Therefore, from this point of view, these two models were the most efficient.

Regarding the analysis of residuals, the graphs in Figure 10 shows two examples of residuals distribution of PSSRa and Vi_{opt}. The linear trends observed in the graphs confirms the "normal" nature of the residuals.

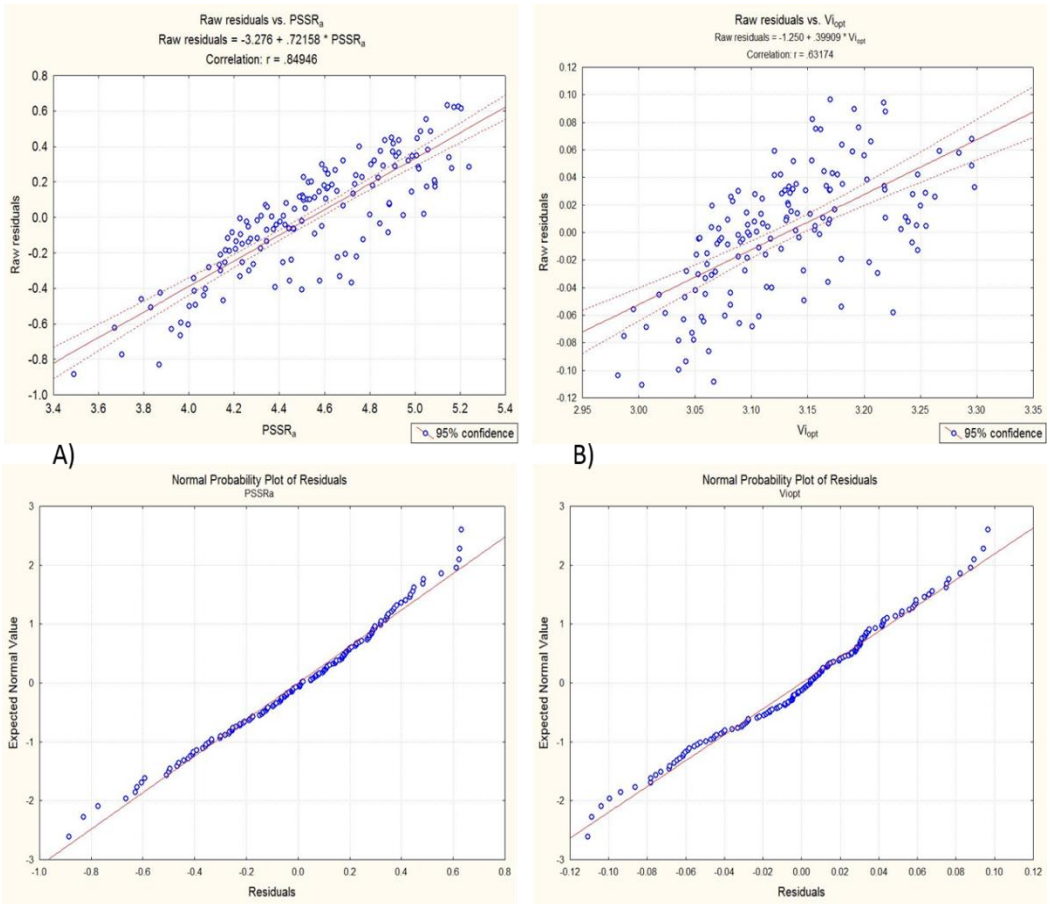

Figure 10. Distribution of residuals of PSSRa (top A) and Vi_{opt} (top B). *The points almost completely aligned on the straight lines (bottom A and B) confirm the normal distribution of the residuals.*

Discussion

This study allowed to evaluate the relationships between hyperspectral data acquired from leaves of ancient wheat landraces using a spectroradiometer and the physiological plant growth measured in terms of plant height.

The variability observed in the hyperspectral signatures was coherent with plant changes during the growth stages (Figure 7). In particular, variability detected in the green region of the spectral curves was related to the change of leaves colour from green to yellow, indicating that at DoY 131 Perciasacchi plants reached the maximum growth level (Figure 7B). Indeed, the further increase of curves variability observed at DoY 161 (Figure 7C) is coherent with the attainment of the maturation stage of Perciasacchi plants. Similar observations were made based on the trend of the slope of the reflectance curves in the near-infrared region which was coherent with the phenologic stage of the plant in accordance with the results obtained in previous works by Rizzuti *et al*. (2018). Therefore, HVIs can be considered highly related to plant growth.

Moreover, these results along with the trends observed in plant height measures at the chosen DoYs, (Figure 9) allowed to confirm that the stem elongation phase, in particular, was the most active in terms of plant growth and, consequently, the most informative phenologic stage for the evaluation of growth parameters. These observations are not surprising as the stem elongation is an important growth trait (Slafer *et al*., 1995) which has been targeted in other works to improve wheat yield (Miralles *et al*., 2000).

Among the analyzed HVIs, Slope, Vi_{opt}, PSSRcar and PSSRa showed the highest correlations with the average of plant height, here considered as growth indicator. In addition, based on the results of the analysis of residuals, the assumptions taken in the procedures for construction of the four statistical models described in this chapter were fulfilled. The results obtained confirm the strong relationships of leaf nitrogen content-related indices (in this case Vi_{opt}) and pigment-related indices (PSSRa, PSSRcar) with plant growth. Relationships between plant nitrogen intake and leaf chlorophyll content have been assessed in previous studies (Peltonen *et al*., 1995), therefore the correlation of both types of indices with plant growth is coherent. Interestingly, the index Slope, defined and evaluated in this work, showed a strong correlation with wheat growth. Further investigations are still needed, through correlation studies involving plant physiological and biochemical components, in order to

confirm the relationship observed in this study with the possibility of optimizing the spectral correlation by implementing new wavelengths ranged between 800 – 1300 nm.

In conclusion, further studies have been carried out, and described in the next chapters, in order to evaluate the relationships of wheat growth-related HVIs, including Vi_{opt}, PSSRa, PSSRcar and Slope with the composition of the fungal endophytic community (Chapter 3) and bacterial plant-growth promoting activity (Chapter 4) associated with ancient wheat landraces.

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Chapter 3

Study of the endophytic fungal community in ancient tetraploid wheat

Abstract

The fungal community composition and structure of two ancient tetraploid wheat varieties, native to the Sicilian territory, Perciasacchi (winter wheat) and Tumminia (spring wheat) were investigated. The results showed a predominant presence of Ascomycetes and Basidiomycetes fungi which were mainly represented by the genera *Alternaria, Fusarium, Mycosphaerella, Filobasidium, Cystofilobasidium, Cryptococcus, Leucosporidium, Dioszegia, Puccinia, Sporobolomyces, Aureobasidium, Cladosporium, Holtermanniella* and *Gibberella*. Principal Coordinates Analysis (PCoA) and Linear discriminant analysis Effect Size (LEfSe) showed that *Aureobasidium, Leucosporidium* and *Puccinia* differentiated between the two wheat varieties. In addition, the microbial association analysis suggested that some endophytic taxa play and important role within the wheat fungal community. Genera such as *Cryptococcus* and *Cystofilobasidium* were shown to have a consistent antagonistic activity against *Gibberella* species, while, *Acremonium* and a group of unidentified ascomycetes had a mutual exclusion relationship with the genus *Puccinia.* Since both *Gibberella* and *Puccinia* contain several phytopathogenic species that cause economically important diseases in wheat, the detected interactions may indicate a microbial-mediated resistance of wheat varieties. In addition, the relationships between hyperspectral vegetation indices (HVIs) and the average of plant height obtained from morphometric and proximal sensing measures with the endophytic fungal genera detected in this study were investigated. According to Spearman correlation, several genera, including *Alternaria*, showed direct relationships with the average of plant height and a group of HVIs. These results suggest the presence of beneficial endophytic species belonging to some fungal genera detected in this study and the need to deepen the relationships between HVIs and the plant-associated fungal community in order to identify more indices to be used for improving agriculture management.

Introduction

Plant-associated microorganisms, collectively referred to as the plant microbiota, are known to have an influence on the plant's physiological development and response to environmental changes such as stress tolerance, disease resistance, and interactions with competitors and predators (Berendsen *et al*., 2012; Lugtenberg and Kamilova, 2009). Endophytes are commonly defined as microorganisms that can be isolated from surface-disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant (Petrini, 1991). They have recently gained a center stage in both basic and translational science due to their potential role as powerful biocontrol agents (Abdelfattah *et al*., 2018; Massart *et al*., 2015; Dorworth and Callan, 1996).

Wheat (*Triticum sp*.) is a global staple crop adapted to a wide variety of environmental conditions, including more marginal areas (Salmon and Clark, 1913). In Italy, the production of durum wheat (*Triticum durum*) accounts for more than 50% of total European production (European Commission, Eurostat and DG Agriculture and Rural Development). Intriguingly, the production of this crop is mainly concentrated in hot and dry southern regions, including Sicily (https://gain.fas.usda.gov, http://dati.istat.it). Recently, the valorization of local ancient wheat varieties acquired a great importance due to their peculiar organoleptic properties which fueled an expanding craft milling and bakery landscape (Jankielsohn and Miles, 2017; Shewry and Hey, 2015). For example, in Sicily, the importance of local ancient landraces of tetraploid wheat such as Perciasacchi and Tumminia is raising due to the quality of their flour as well as to their low nutritional requirements and ability to grow in dry environmental conditions typical of the Sicilian region. This has sparked a scientific curiosity about the molecular determinants of such adaptive capacities.

In this regard, the possibility of finding microbial strains capable of improving wheat productivity and tolerance to biotic and abiotic factors has motivated many scientific studies in the last decade (Hubbard *et al*., 2014; Velázquez-Sepúlveda *et al*., 2012; Larran *et al*., 2007; Coombs and Franco, 2003). More recently, advances in sequencing and computational applications, such as metabarcoding (Taberlet *et al*., 2012), have enabled scientists to characterize the microbial diversity at an unprecedented depth (Ofek-Lalzar *et al*., 2014; Turner *et al*., 2013). Most of these studies focused on the bacterial components while little is known about fungi although it is becoming increasingly clear that the fungal microbiota plays

a critical role in plant growth, development and stress tolerance (Abdelfattah *et al*., 2018; Nicolaisen *et al.*, 2014; Shendure and Ji, 2008).

Yet, it remains fundamentally unclear whether locally-adapted wheat varieties are capable of recruiting a distinct microbiota and, if so, to what extent this differential recruitment is influenced by the environment and the host genotype. As a first step towards deciphering the contribution of wheat microbiota to crop adaptation and yield, in this study we report a molecular characterization of the composition of the fungal endophytic communities of two ancient Sicilian tetraploid wheat varieties, the winter type Perciasacchi and the spring type Tumminia, grown in two agricultural fields. Finally, the correlations between the average of plant height (APH), the hyperspectral vegetation indices (HVIs), measured and studied in Chapter 2, and the taxonomic information obtained through the HTS approach was evaluated using statistical methods.

Materials and methods

Plant sampling

Plant sampling was carried out within the sampling scheme described in "Experimental sites and sampling schemes" (Chapter 1). Moreover, among the sampling points placed in the fields, 23 and 27 plots were selected for sampling in Field 1 and 2 respectively, based on the results of proximal sensing measures with the aim of including the whole biodiversity available. During the heading phase of the two wheat varieties, a total of 50 samples were collected from the selected sampling plots. Each sample consisted of ten plants including their roots. Samples were transported to the laboratory and stored at 4°C before processing.

Sample preparation

The aerial part of the plant was cut using sterile scissors and a stem portion of approximately 15 cm above the crown was kept for sap extraction. Stem and root samples were surface-sterilized using 5% Sodium hypochlorite (NaClO) and rinsed in sterile water. Sap extraction was carried out using a new method, the CIHEAM-IAMB patented 'Method for the extraction of sap from plant material and apparatus for carrying out the method' (https://patents.google.com/patent/WO2017017555A1/en). This method of extracting plant sap from vessels and xylem using the pressure of a syringe has the advantage of reducing

plant components in the final extract that can inhibit enzyme activity, adversely affecting the results of the amplification protocol. One ml of Phosphate-buffered saline (PBS) solution was inserted into one terminal of the plant stem using a syringe. PBS with the sap extracts was collected from the other terminal of the plant stem in a sterile 1.5 ml tube, which was later used for plating and DNA extraction. Afterwards, 28 samples were randomly chosen, seven from each half of each field (14 from each wheat variety), for high throughput sequencing (HTS).

Culture conditions of wheat endophytic fungi and DNA extraction

Endophytic fungi were cultured by aseptically transferring 100 µl of the wheat sap extract on 9 cm Petri's dishes filled with semi-selective Nutrient Yeast Dextrose Agar (NYDA) medium (glucose 10 g/l, yeast extract 5 g/l, nutrient broth 8 g/l, agar 18 g/l, streptomycin sulphate 250 mg/l and ampicillin 250 mg/l) (Janisiewicz, 1988). The inoculated dishes were incubated for 48 - 72 hours at 26°C. After the incubation time, a small portion of the growing mycelium was transferred to new Potato Dextrose Agar (PDA) medium. A small portion of the grown mycelium was transferred from each plate in a unique sterile 1.5 ml tube, using a handle, and stored at -20°C. Finally, DNA extraction from 400 µl of wheat sap extract and the mixed fungal mycelium was performed using NucleoSpin® Plant IIextraction kit (Macherey-Nagel) following the manufacturer's instructions and doubling the pre-lysis and lysis incubation times.

Metabarcoding analysis

Samples for the metabarcoding analysis were grouped into the following four categories including seven biological replicates: P1 (Perciasacchi grown in Field 1), T1 (Tumminia grown in Field 1), P2 (Perciasacchi grown in Field 2) and T2 (Tumminia grown in Field 2).

Amplifications of the fungal ribosomal Internal Transcribed Spacer 2 (ITS2) region were performed using the forward primer ITS86F (Turenne *et al*., 1999) and mix reverse primers ITS4-Mix 1, ITS4-Mix 2, ITS4-Mix 3, and ITS4-Mix 4 (Tedersoo *et al*., 2014, 2015). Amplifications of the ITS2 region were performed using KAPA HiFi Hot Start ReadyMix kit (KAPA Biosystems, USA), under a temperature profile of 95°C for 3 min, 35 cycles at 98°C for 20 sec, 56°C for 15 sec,72°C for 30 sec, followed by an elongation step of 72°C for 1 min. PCR products were visualized on 2% agarose gel in order to verify the successful

amplification and the absence of contamination. PCR purification was performed using AgencourtAMPure XP beads kit and following the user manual instructions (Beckman Coulter, USA). Amplicon indexing was carried out using Nextera XT v2 Index Kit (Illumina, USA) and followed by a second PCR purification as previously described. Amplification products were quantified by fluorimetry using Qubit (Invitrogen, USA) and pooled in equimolar concentrations before sequencing reactions in MiSeq (Illumina, USA) according to the manufacturer guidelines (support.illumina.com). Datasets generated during the current study were deposited and are available at the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA), under the accession number PRJNA449228 (www.ncbi.nlm.nih.gov/bioproject/ PRJNA449228).

Sequencing data analysis

Raw reads were trimmed using Trimmomatic v. 0.32 (Bolger et al., 2014), using a sliding window of 6 bases, Q-score average of ≥ 20, and a minimum sequence length of 150 bp. The paired-end reads were then assembled using PANDAseq Assembler (Masella *et al*., 2012) setting a minimum sequence length value of 150 bp and a minimum overlapping value of 20 bp. Reads were then checked and filtered for chimeric sequences using VSEARCH v.1.11 (Rognes *et al.*, 2016) and the UNITE dynamic database released on November 20, 2016 (https://unite.ut.ee). The same database was also used for creating Operational Taxonomic Units (OTUs) with a similarity threshold of 99% and for taxonomy assignments with BLAST method (Altschul *et al*., 1990) as implemented in QIIME v. 1.9.1. (Caporaso *et al*., 2010). This taxonomical information was collapsed to describe the fungal community at the genus, family, order, class, and phyla level.

Downstream analyses

The downstream analyses were conducted using QIIME 1.9.1 pipeline (Caporaso *et al*., 2010) as described by Abdelfattah and co-workers (Abdelfattah *et al*., 2017). The OTU table was normalized by rarefaction to an even depth of 57,623 for cultured mycelium and 42,448 for sap extract samples in order to reduce sample heterogeneity as well as to keep samples with an acceptable number of sequences to be used in statistical and taxonomic analyses. Fungal richness and abundance were calculated through alpha-diversity analyses determined by Shannon's Diversity, Simpson, Chao1, and Observed OTUs indexes. The diversity results

were then compared using a nonparametric two-sample t-test, the p-values were calculated through 999 Monte Carlo permutations. Moreover, beta diversity analysis was performed using Bray Curtis dissimilarity metric and the results were used to conduct Principal Coordinates Analysis (PCoA). PCoA graphs were also implemented with the taxonomical information and plotted on a 3D graph using EMPeror (Vázquez-Baeza *et al.,* 2013). Beta diversity results were used to compare groups of samples (fields, wheat varieties and sample categories) using Permanova analysis. Finally, differentially abundant taxa between the two fields, wheat varieties and the four sample categories were detected using the Linear discriminant analysis Effect Size (LEfSe) (Segata *et al.,* 2011) setting a p-value threshold for the factorial Kruskal-Wallis test and for the pairwise Wilcoxon test of 0.05 and the logarithmic Effect Size (LDA) cut-off > 2.

Fungal association network

Inferred fungal associations (co-occurrence and mutual exclusion) within each wheat variety were computed using the CoNet (v1.1.1. beta) plugin within Cytoscape (v3.6.1). The associations of OTUs present in at least 20 samples were identified using an ensemble of correlation (Spearman and Pearson coefficients) and distance (Bray–Curtis and Kullback–Leibler dissimilarity measures) metrics. For each association metric and each edge, 100 renormalized permutation and bootstrap scores were generated following the ReBoot procedure (Faust *et al.*, 2012). The measure-specific p-values from multiple association metrics were merged using the Simes method (Sarkar and Chang, 1997) and all edges with p values above 0.05 were discarded. False-discovery rate correction was performed using Benjamini–Hochberg multiple testing correction (Benjamini and Hochberg, 1995). Only 1000 top- and 1000 bottom-ranking edges from each association measure were kept in the network analysis, and only edges supported by at least two of the four association metrics were retained in the final network inference of associations among taxa.

Correlations analysis of the endophytic fungal communities with wheat-associated hyperspectral and morphometric parameters

The non-parametric Spearman correlation analysis was used to evaluate the relationships between the average of plant height (growth indicator), the hyperspectral vegetation indices (spectral bioindicators) selected in Chapter 2 (Slope, Vi_{opt}, PSSRcar e PSSRa) and the fungal endophytic communities associated to two ancient wheat landraces under study, grown in the two fields at DoY 131. Statistical analyses were performed using Statistica v.8 in a Windows environment (StatSoft inc.).

Results

Sequencing data processing

HTS generated a total of 1,509,410 and 4,492,327 unpaired reads from fungal mycelium and wheat sap extract respectively. After trimming, pairing, quality and chimera filtering, 687,093 and 2,330,956 high-quality fungal sequences were retained from both groups of samples respectively. The resulting sequences were then assigned to 2,943 OTUs in mycelium samples

and 6,885 OTUs in sap samples. A summary of the sequencing results is shown in Table VII and Table VIII.

Table VII. Summary of HTS results of cultured mycelium samples. *Sequencing results obtained from the analysis of the DNA extracted from cultured mycelium belonging to the four categories: P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2.*

Category	**N. reads**	**Total OTUs**	**Rarefied OTUs**
P1	83,331	927	927
T1	127,221	960	960
P2	57,623	615	615
T2	84,962	753	753
Rarefaction depth 57623			

Table VIII. Summary of HTS results of sap extract samples. *Sequencing and alpha diversity results obtained from the analysis of the DNA extracted from wheat sap extract belonging to the categories: P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2.*

Sequencing results				**Alpha diversity metrics**			
Category	**N. reads**	**Total OTUs**	**Rarefied OTUs**	**Simpson**	**Shannon**	**Chao1**	**Observed Species**
P1	459,150	5,454	3,181	0.95	5.62	1249.40	651.52
T1	299,769	2,646	1,805	0.96	5.67	1252.82	555.92
P2	566,026	4,047	2,815	0.92	4.61	1072.07	507.20
T2	949,383	6,612	3,799	0.93	5.15	908.93	498.35
Rarefaction depth 42448							

Cultured endophytic fungi from wheat

The sequencing of the ITS2 region of the endophytic fungi isolated in culture plates provided us with the taxonomical composition of the cultivable wheat endophytic fungi (Figure 11). A total of 12 species belonging to 10 genera were identified. The number of genera per sample varied from four to nine. Ascomycota was the representative phylum of all the cultured fungal genera where *Mycosphaerella*, *Acremonium*, *Cladosporium*, *Aureobasidium*, and *Fusarium* were shared between all the tested samples. Other genera were detected only in some sample categories: *Fusarium* and *Alternaria* (P1, T1 and T2), *Microdochium* (P1, T1) and *Monographella* (T1, T2).

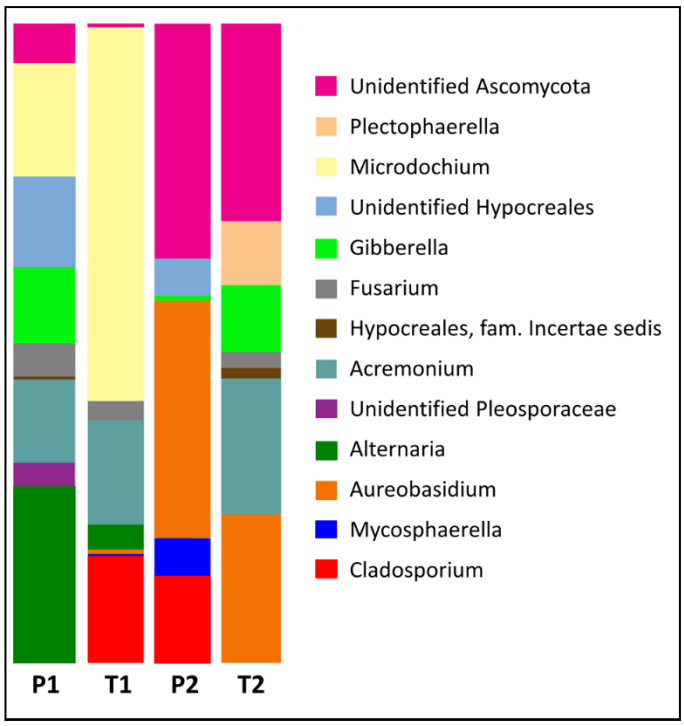

Figure 11. Taxonomical composition of cultured endophytic fungi. *Genera distribution among the four sample categories (P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2) of the endophytic fungi grown in plate culture conditions and analyzed using HTS.*

Wheat endophytic fungal community composition

The taxonomical assignment of the OTUs obtained from the HTS of wheat sap samples elucidated the composition of the wheat endophytic fungal community among the four sample categories (P1, T1, P2 and T2). Overall, 28 OTUs belonging to 26 fungal genera were identified (Figure 12).

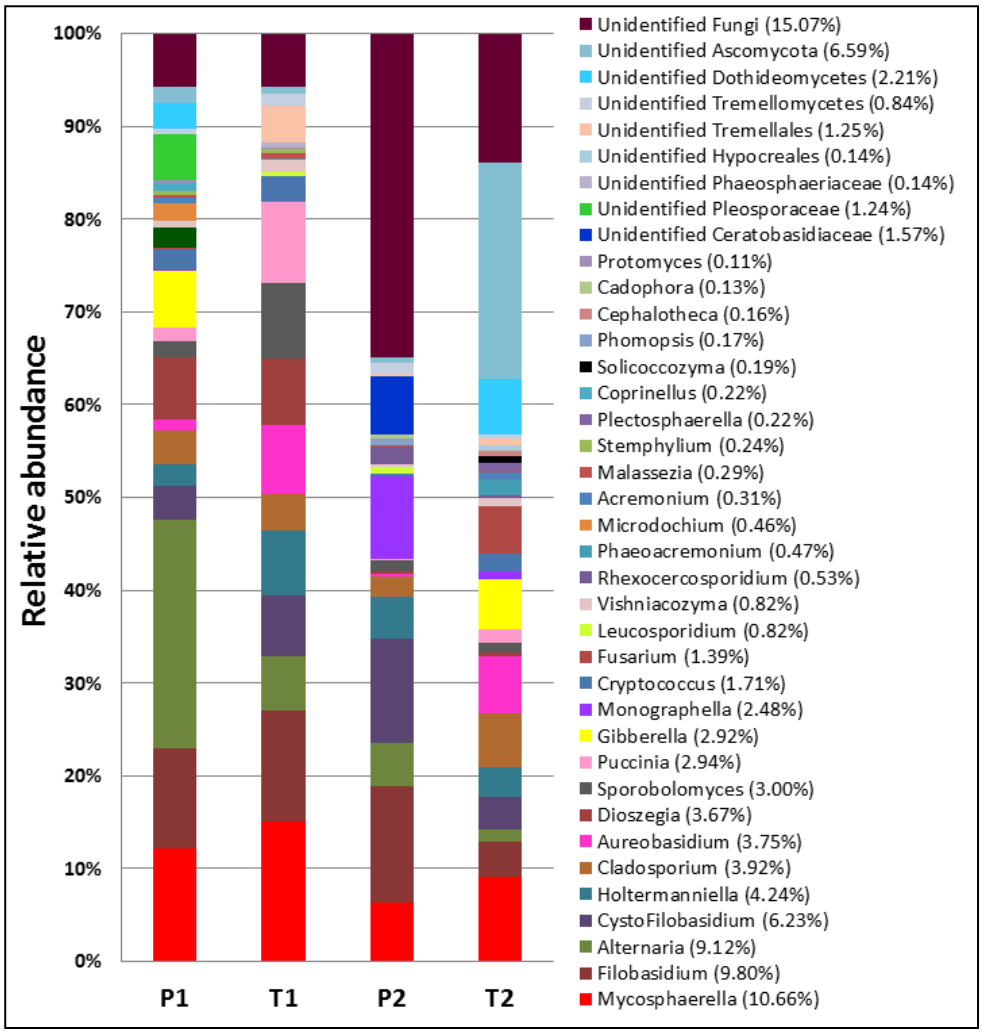

Figure 12. Endophytic fungal richness in wheat. *Distribution and relative abundance of the most abundant fungal genera detected among the four sample categories and resulted from the analysis of wheat sap samples. Average of relative abundance for each genus is reported in parentheses. P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2. Mean values of relative abundance are indicated within the parenthesis.*

However, the relative abundance (RA) of the detected fungal taxa showed some differences between sample categories already at the phylum and class levels (Figure 13). For example, members of Ascomycota were predominant in P1 and T2 with 60% and 68% of RA, respectively. Conversely, Basidiomycota were the most abundant in P2 and T1 with RA values of 39% and 59%, respectively. Moreover, field 2 showed the highest number of unidentified fungal sequences, in particular in the T2 category. *Dothideomycetes* and *Tremellomycetes* were the main representative fungal classes in all the samples with an average of relative abundance of 30%, followed by *Sordariomycetes* (7.5%), *Agaricomycetes* (2.5%), *Microbotryomycetes* (4.4%), *Pucciniomycetes* (3.4%), *Leotiomycetes* (1.0%), *Ustilaginomycotinaincertae-sedis* (0.4%), and *Taphrinomycetes* (0.2%). At the genus level, the following fungi were detected in all sample categories: *Alternaria*, *Mycosphaerella*, *Cladosporium*, *Filobasidium*, *Holtermanniella*, *Cystofilobasidium* and *Cryptococcus*.

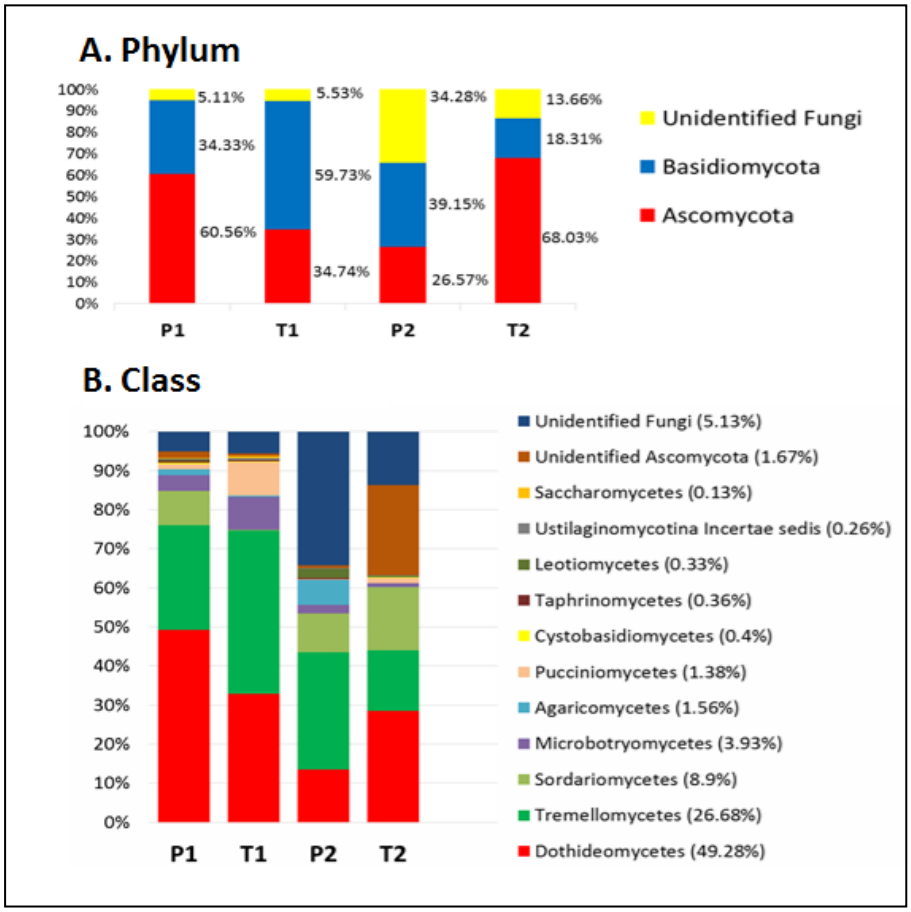

Figure 13. Structure and richness of the wheat endophytic fungal community. *Richness of fungal phyla (A) and classes (B) resulted from downstream analyses of the HTS performed on DNA extracted from wheat sap. Average of relative abundance for each class is reported in parentheses. Sample categories: P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2.*

Endophytic fungal richness and diversity

The number of observed fungal OTUs per sample varied between 2,646 to 6,612 OTUs. Alpha diversity indices showed that samples collected from Field 1 had a higher fungal diversity compared to those from Field 2 (Table VIII). The two-sample t-test based on Shannon index, revealed a significant difference between the two fields (p-value = 0.02). However, there was no significant difference neither between the same variety grown in different fields nor between the two varieties grown within the same field.

The PCoA plots, showing beta-diversity results, evidenced that samples collected from the two fields tended to segregate separately. In addition, the two wheat varieties clustered into different groups within each field (Figure 14). In agreement with PCoA and alpha diversity results, statistical comparisons using non-parametric Permanova test showed a significant variation of the fungal communities between the two fields (p-value = 0.001). In addition, the fungal communities showed to be significantly different between the wheat varieties under study (p-value = 0.044). A significant difference was still observed when the same variety grown in different fields where compared (p-value = 0.002 and 0.005, respectively).

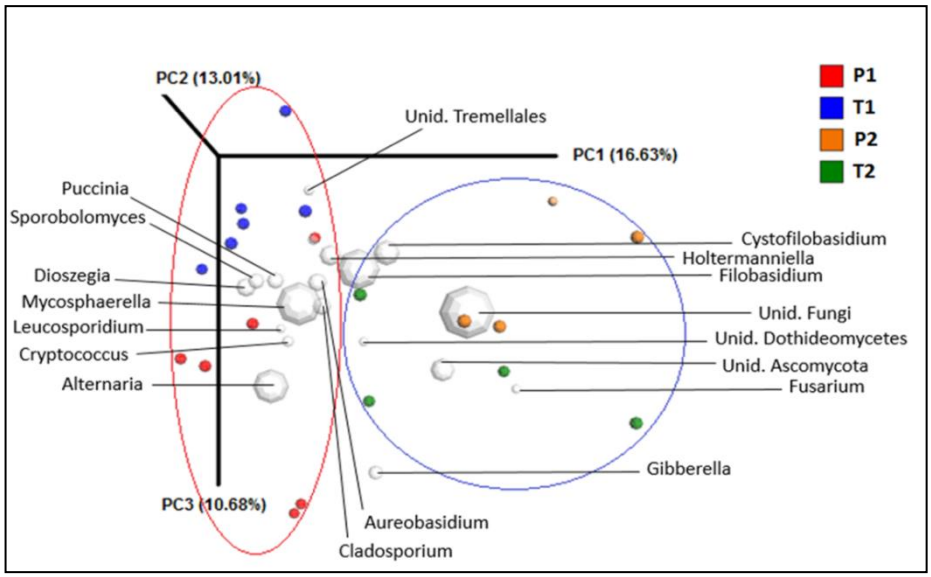

Figure 14. Principal Coordinates Analysis (PCoA). *Integration of taxonomy in the PCoA graph showing the distribution of the 18 most abundant endophytic fungal genera among the four sample categories (white spots). The taxonomic plot weight and the distance from the sample plots are proportional to the relative abundance of each genus. Red circle: cluster of Field 1 samples; blue circle: cluster of Field 2 samples. The different colored spots evidence the sample categories under study: P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2.*

Some of the detected taxa had a significant different relative abundance in the investigated fields and varieties. In particular, the genera *Mycosphaerella*, *Dioszegia*, *Filobasidium*, *Protomyces* and *Alternaria* varied significantly between fields (Figure 15), while *Aureobasidium*, *Leucosporidium* and *Puccinia* varied between varieties (Figure 16).

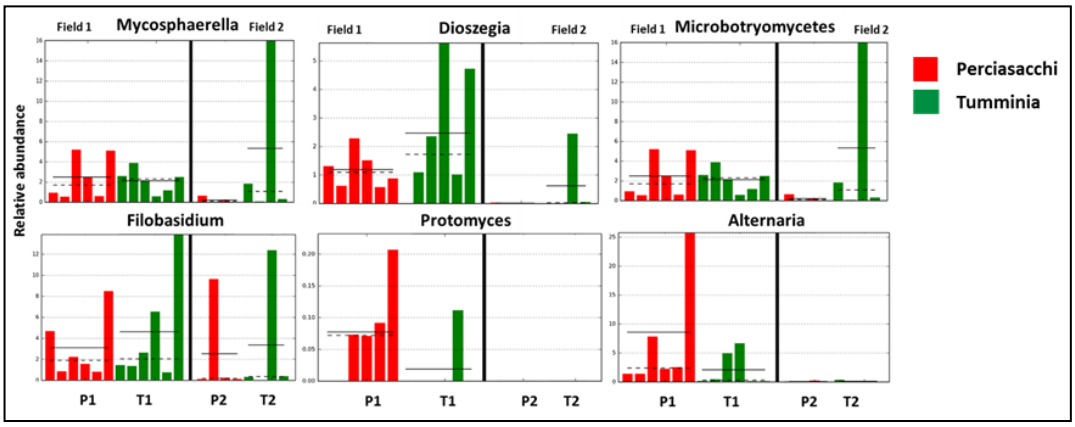

Figure 15. Differentially abundant genera between the two fields and the four sample categories. *Histograms evidencing significant differences calculated by LEfSe analysis with a p-value < 0.05 and LDA cut-off >2. P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/Field 2; T2= Tumminia/Field 2. Category means (straight line) and medians (dotted line) are evidenced.*

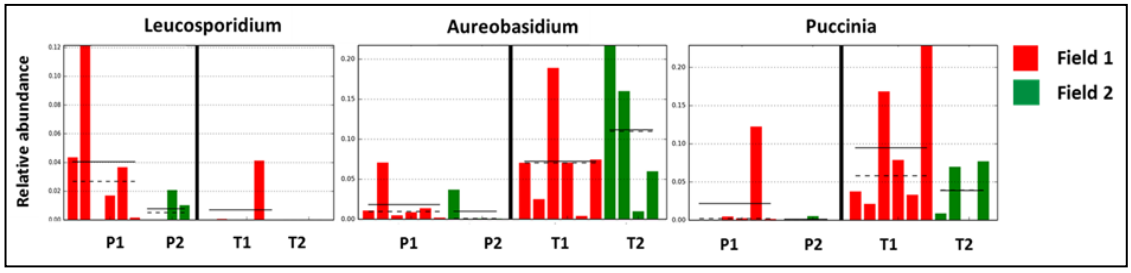

Figure 16. Discriminant genera between the two wheat varieties. *Differential genera between Perciasacchi (red) and Tumminia (green), resulted by LEfSe analysis with p-value < 0.05 and LDA cut-off > 2. Histograms represent differential taxa distribution between the two wheat varieties and among the four sample categories (P1= Perciasacchi/Field 1; T1= Tumminia/Field 1; P2= Perciasacchi/ Field 2; T2= Tumminia/Field 2). Category means (straight line) and medians (dotted line) are evidenced.*

Fungal interaction

The co-occurrence and mutual exclusion of specific fungal OTUs was analyzed for each wheat variety. The resulting networks, after statistical calculations and removal of unstable edges/links, were characterized by 132 and 134 nodes (OTUs) linked with 420 and 583 edges, and a clustering coefficient of 0.606 and 0.731 in Perciasacchi and Tumminia respectively (Figure 17). Overall the interactions between fungal phylotypes were characterized by a higher number of co-occurrences (401 and 510) compared to mutual exclusions (19 and 73) in Perciasacchi and Tumminia respectively. In both cultivar, *Cryptococcus*, *Cystofilobasidium*, and *Holtermanniella* were the dominant genera with a consistente co-occurrences interaction between each other as well as within each genera. *Cryptococcus* and *Cystofilobasidium* had mutual exclusion relationships with *Gibberella* species. *Gibberella* on the other hand, together with *Acremonium* and a group of unidentified ascomycetes had a mutual exclusion interaction with *Puccinia*. Only in Perciasacchi, *Cryptococcus* excluded *Sporobolomyces.*

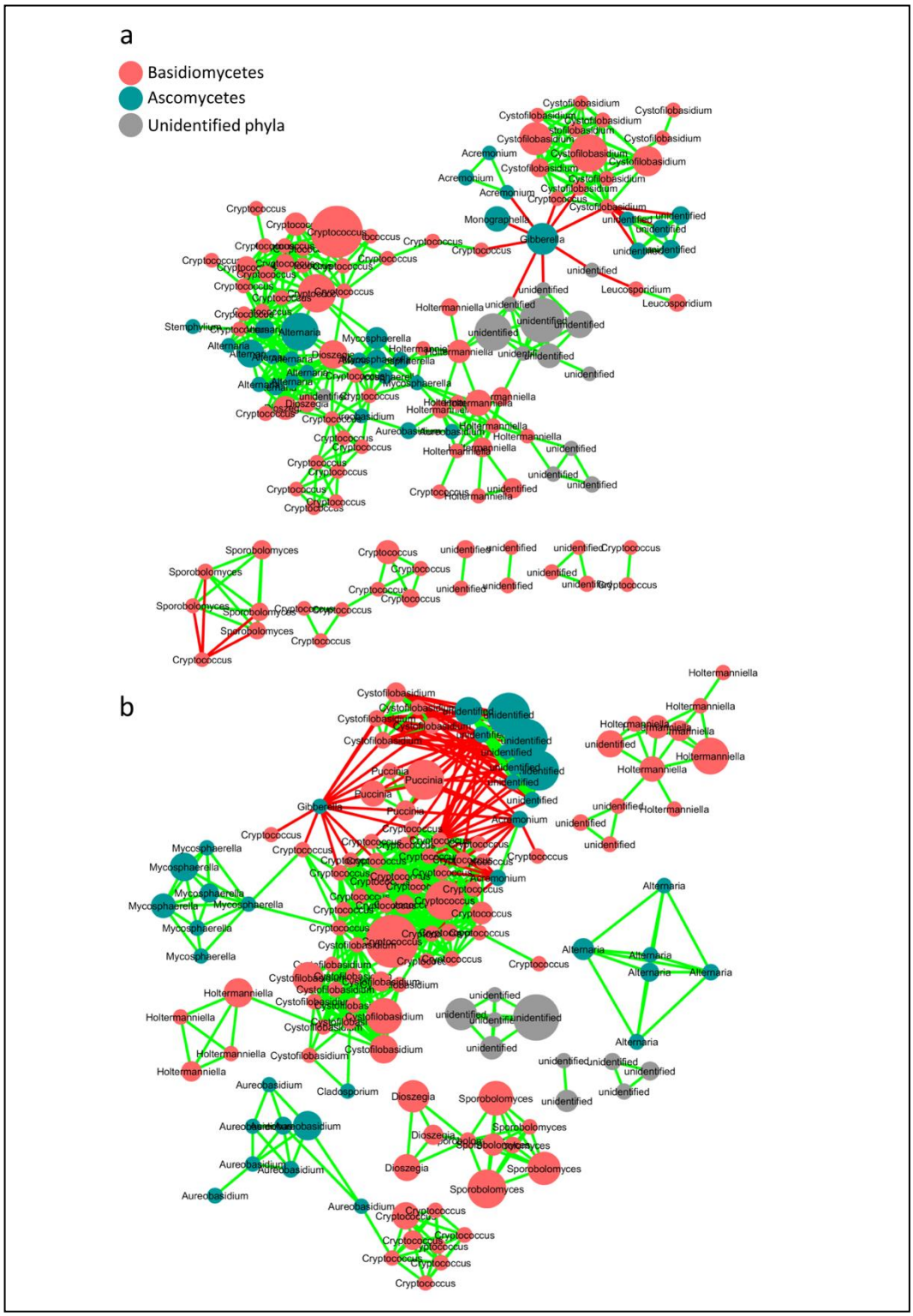

Figure 17. Endophytic fungal interactions. *Microbial association network showing the interactions (co-occurrence and mutual exclusion) represented by green and red links, respectively in Perciasacchi (a) and Tumminia (b) varieties. The size of the nods indicates OTUs abundance. Node colours are used to differentiate fungal phyla. Interactions were calculated by CoNet and visualized in Cytoscape 3.6.*

Correlation study between hyperspectral vegetation indices, plant height measures and fungal taxonomic data

The non-parametric Spearman analysis showed significant correlations between the average of plant height (APH), the four selected indices Slope, Vi_{opt}, PSSR car and PSSRa and the taxonomic data along with the relative abundancies of the fungal endophytic genera detected using HTS (Table IX).

Table IX. Spearman correlation. *Summary of the significant correlations (p-level ≤ 0,05) resulted from the non-parametric Spearman correlation of average of plant height (APH), hyperspectral vegetation indices (HVIs) and endophytic fungal genera detected using HTS.* ***Bold:*** *most recurrent fungal genera related to the plant-growth parameters analyzed.*

Parameter & fungal genus	Valid N	Spearman R	t	p-level
Average plant height				
APH & Other	**12**	**-0.615**	**-2.46**	**0.03342**
APH & Alternaria	**12**	**0.628**	**2.55**	**0.02875**
APH & Protomyces	**12**	**0.591**	**2.32**	**0.04287**
APH & Taphrina	12	0.583	2.27	0.04665
APH & Solicoccozyma	**12**	**-0.734**	**-3.42**	**0.00654**
APH & Bullera	**12**	**0.658**	**2.77**	**0.01995**
HVIs				
Slope & Other	**20**	**0.510**	**2.51**	**0.02163**
Slope & Acremonium	20	0.598	3.16	0.00539
Slope & g_Filobasidium	20	-0.561	-2.87	0.01009
Slope & g_Solicoccozyma	**20**	**0.487**	**2.37**	**0.02935**
Slope & p_Basidiomycota	20	-0.518	-2.57	0.01940
Vi_{opt} & g_Zymoseptoria	20	0.503	2.47	0.02390
Vi_{opt} & g_Alternaria	**20**	**0.642**	**3.56**	**0.00226**
Vi_{opt} & g_Microdochium	20	0.521	2.59	0.01850
Vi_{opt} & g_Protomyces	**20**	**0.486**	**2.36**	**0.02988**
Vi_{opt} & g_Bannoa	20	0.518	2.57	0.01928
Vi_{opt} & g_Mastigobasidium	20	0.457	2.18	0.04254
Vi_{opt} & g_Bullera	**20**	**0.447**	**2.12**	**0.04807**
PSSRa & g_Zymoseptoria	20	0.449	2.13	0.04714
PSSRa & g_Alternaria	**20**	**0.450**	**2.14**	**0.04674**
PSSRcar & g_Zymoseptoria	20	0.497	2.43	0.02569
PSSRcar & g_Alternaria	**20**	**0.471**	**2.26**	**0.03623**
PSSRcar & g_Microdochium	20	0.494	2.41	0.02699
PSSRcar & g_Protomyces	**20**	**0.446**	**2.12**	**0.04858**
PSSRcar & g_Bannoa	20	0.488	2.37	0.02912

APH resulted significantly and directly correlated with the genera *Bullera, Alternaria, Protomyces* and *Taphrina,* whereas *Solicoccozyma* and a group of unidentified OTUs belonging to the "Other" group showed an inverse correlation.

Regarding the hyperspectral bioindicators, with the exception of *Taphrina*, all fungi that showed correlation with APH were also significantly correlated with the selected group of indices. In addition, Slope showed significant correlations with *Acremonium*, *Filobasidium* and *Basidiomycota*; whereas, Vi_{opt}, PSSRa and PSSRcar were correlated with *Microdochium*, *Bannoa, Zymoseptoria* and *Mastigobasidium*.

Discussion

This study has demonstrated the limits of culture-dependent methods for characterization of the endophytic fungal communities. Metabarcoding analyses from crude sap extract allowed the detection of 26 genera belonging to Ascomycota and Basidiomycota, while fungal isolations allowed the detection of only ten genera belonging to Ascomycota. Since isolations were carried out using one culture medium under one condition, a broader range of culturing conditions may be required to capture greater proportion of wheat endophytes. Nevertheless, nine out of 12 fungal species grown in plate cultures were also detected in the wheat sap extracts, therefore a good correspondence was obtained between the fungal taxa identified by the two methods. The variability in detection of the two methods could be due to bias of the culturing technique, which may have favored some taxa over others. Alternatively, the choice of primers for HTS analyses may have impact on the numbers of detected taxa (Tedersoo *et al.,* 2015).

The variations observed in the endophytic fungal communities between each wheat variety grown in the two fields could be explained by the different locations and agronomic conditions at the two field sites. Field 1 had been to crop rotation with legumes, while Field 2 was not cultivated for five previous years. Previous studies have reported that geographical location as well as crop management practices can affect the composition of soil microbial communities and, as consequence, the composition of endophytic microbial communities (Göre and Bucak, 2007; Sapkota *et al.,* 2017; Soman *et al.,* 2017).

The fungal communities were different between the two wheat varieties. This is not surprising since host genotype is considered to be a major factor determining the composition of endophyte communities (Sapkota *et al.,* 2015). These results highlight the importance of

investigating microbial diversity of ancient crop varieties as possible sources of beneficial microorganisms.

Alternaria, *Cladosporium*, *Sporobolomyces*, *Dioszegia* and *Cryptococcus* are reported to be ubiquitous, and they have been detected in the present study as well as in the phyllospheres and grain of several commercial wheat varieties (Nicolaisen *et al.*, 2014; Sapkota *et al.*, 2015). On the other hand, genera such as *Filobasidium*, *Holtermanniella* and *Cystofilobasidium* were detected in Perciasacchi and Tumminia, but have not been detected in the phyllospheres of modern cultivars. Conversely, *Pyrenophora*, *Epicoccum*, *Phoma* and *Sphaeosphaeria* belonging to core OTUs in the phyllospheres of modern wheat cultivars (Nicolaisen *et al.*, 2014) were not found in the present study. Differences between ancient and modern cultivars may be the consequence of different breeding histories as well as cultivar selection and/or growing conditions. Further research comparing varieties with contrasting pedigrees is necessary to firmly define taxa shared, or differentiating between, wheat genotypes, as previously reported for other crop species (Liu *et al.*, 2018).

Despite the presence of fungal genera comprising important plant pathogenic species, no disease symptoms were observed in the collected samples from both fields. The presence of non-pathogenic fungal strains and plant resistant genes may account for this observation. However, an important contribution of beneficial fungal strains in keeping a plant health condition is very likely. In fact, the analysis of the association networks suggested the existence of key endophytes playing and important role within the wheat fungal community. For instance, Basidiomycete endophytes such as *Cryptococcus* and *Cystofilobasidium* were the predominant genera and seemed to have a consistent antagonistic activity against *Gibberella* species. Similarly, *Acremonium* and a group of unidentified ascomycetes had a mutual exclusion relationship with the genus *Puccinia.* Since both *Gibberella* and *Puccinia* include several phytopathogenic species that cause economically important diseases in wheat, the detected interactions may indicate a microbial-mediated resistance of wheat varieties. This hypothesis needs to be confirmed with further experiments aimed to verify the antagonistic behavior between strains belonging to the above-mentioned genera. Furthermore, the fact that *Holtermanniella* and *Cystofilobasidium* were detected in the present study in ancient cultivars, and have not been reported to colonize modern wheat cultivars, may suggest a negative impact of breeding and domestication on the beneficial native microbiome.

Moreover, the significant correlation observed between the fungal genera identified in this study, the hyperspectral vegetation indices and the morphologic measures (average of plant

height) associated with plant growth showed a direct correlation with the genus *Alternaria*. This result is particularly surprising as most *Alternaria* species as well documented plant pathogens and mycotoxins producers (Müller and Korn, 2013). However, some endophytic strains belonging to this genus are reported to produce antimicrobial compounds (De Siqueira *et al*., 2011). Considering the ubiquitarian nature of this genus, which has been isolated form plant tissues including wheat, it is likely that it might include beneficial strains (Ofek-lalzar *et al*., 2016). Similar hypotheses might be done regarding the other fungal genera resulted to be correlated with plant height and the HVIs.

In conclusion, the results obtained in this study expanded our knowledge on the endophytic fungal community associated with ancient wheat cultivars and allowed the formulation of hypotheses on their role in the plants. However, further studies, including the comparison between ancient and modern wheat microbiota, are needed in order to clarify the role played by specific fungal taxa and facilitate their exploitation as alternative means to improve plant health.

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Chapter 4

Isolation and screening of beneficial bacteria associated to ancient tetraploid wheat landraces

Abstract

The plant microbiota has been widely studied in the last decades for its influence on plant health and physiology. Indeed, the possibility of using beneficial microorganisms to improve plant growth and health might be useful for developing more environmentally friendly approaches to improve agriculture sustainability. Most of them are based on plant-growth promoting rhizobacteria (PGPR) that proved to actively benefit plant health in economically important crops. Among them, wheat is grown almost globally and tetraploid wheat varieties have a great importance in Italy. It is grown mainly in the southern regions where there is a raising valorization of ancient tetraploid wheat landraces considered an important source of genetic and microbial diversity. In this work, several bacterial strains were isolated from plant tissues of two ancient tetraploid wheat landraces native of the Sicilian territory, Perciasacchi (winter wheat) and Tumminia (spring wheat). Moreover, screenings performed *in vitro* using a culture-dependent approach allowed to detect several bacterial colonies performing plant-growth promoting activities. In addition, proximal sensing approach has raised a great interest for the possibility of calculating hyperspectral vegetation indices (HVIs) related to plant physiological and stress conditions. However, the relationships between plant-growth promoting (PGP) activities and HVIs have not yet been studied. Therefore, in the present study statistical analyses were performed to evaluate the correlations between the HVIs selected from Chapter 2 and the PGP activities detected from the screened bacterial colonies. Finally, a set of potential PGPRs was selected among the most active bacterial isolates to be further studied in order to assess their effects on wheat growth *in vivo*.

Introduction

The study of the plant microbiota has raised particular interest in the last years for its influence on host physiology and response to stresses. Indeed, plant microbiota is characterized by a complex network of interactions involving the microorganisms, the host and the environment (Berendsen *et al.,* 2012). In addition, the possibility of manipulating the plant-associated microbial communities in order to improve plant growth and health brought to several studies aimed to investigate the taxonomic composition and the interactions involving the plant microbiota (Berendsen *et al.,* 2012; Bulgarelli *et al.,* 2013). Promising results have been obtained so far in the field of the formulation of biofertilizers and biocontrol products in order to reduce the environmental impact of agricultural systems by using microorganisms able to improve soil fertility and plant defences against phytopathogens (Pandey and Maheshwari, 2007; Karthiba *et al.,* 2010; Kalita *et al.,* 2015).

Among them, plant growth promoting rhizobacteria (PGPR) are widely studied for their ability of promoting plant growth through several mechanisms such as the increase of nutrients availability (e.g. ammonium, phosphorus), production of growth-regulator hormones (e.g. indoleacetic acid), biocontrol activities (e.g. siderophores production) and induction of plant systemic defences (Ahmad *et al.,* 2008; Kumar *et al.,* 2012) . In addition, the study of endophytes is of particular interest for their ability of establishing a mutualistic interaction with the plant (Proença *et al.,* 2017). However, application of beneficial bacteria in the field requires easy-growing isolates in culture conditions along with the possibility of testing their beneficial effects on plant growth (Arora *et al.,* 2011). Therefore, several culture-dependent screening methods have been developed and are available in literature in order to test bacteria isolated from soil and plant material for plant growth promoting activities *in vitro* (Bric *et al.,* 1991; Ahmad *et al.,* 2008; Kumar *et al.,* 2012).

These methods could allow the screening of beneficial bacteria associated to important crops, including wheat. Italian tetraploid wheat production accounts for the 50% of the European production and is concentrated mainly in the southern regions and the islands (Motzo and Giunta, 2007). In addition, in Sicily there is an increasing interest in the valorization of native ancient wheat landraces, in particular Perciasacchi and Tumminia as potential source of genetic and microbial diversity (see Chapter 1). In addition, technologies based on the acquisition of hyperspectral reflectance and their derived vegetation indices (HVIs) proved to

be useful tools for the detection of plant physiological parameters related to biotic and abiotic stress (see Chapter 1). In particular, proximal sensing (see Chapter 1) was used in this project to acquire hyperspectral signatures from the leaves of the two ancient tetraploid wheat landraces, Perciasacchi and Tumminia.

Therefore, the aim of this work was to evaluate the presence of beneficial bacteria associated to Perciasacchi and Tumminia. Culture-dependent approaches were used to isolate bacterial colonies from bulk-soil and plant material and screen them for plant-growth promoting (PGP) activities *in vitro* conditions in order to identify isolates with beneficial effects on wheat growth. Moreover, a correlation between the results obtained from bacterial screenings for PGP activities and the HVIs related to plant growth resulted from the analysis of proximal sensing data, as described in Chapter 2, was carried out in order to evaluate whether HVIs could be used to predict the presence of PGP activities in the field.

Materials and methods

Bacteria isolation

Bacteria to be screened were isolated from bulk-soil, wheat roots surface and stem tissue sap by following the sampling scheme described in "Experimental sites and sampling schemes" section in order to isolate environmental, root-associated and endophytic bacterial strains.

Bacteria from bulk soil were isolated by dissolving 1 g of dried and sieved soil in 10 ml of sterile distilled water. Serial 1:10 dilutions of the soil suspension were prepared until reaching 10^{-4} and 1 ml of each dilution was plated on Nutrient Yeast Dextrose Agar (NYDA) medium added with 0.015% cycloheximide in order to prevent fungal growth. Inoculated plates were incubated at 26°C for 24 h.

Root-associated bacteria were isolated by removing the aerial part of the plant (which was used for hyperspectral measures as described in Chapter 2) and washing the roots in tap water to remove soil residuals. Roots were then rinsed in 0.5% Sodium hypochlorite (NaClO) for 30 sec in order to remove tap water contaminants and rinsed in sterile distilled water. Washed roots were dried using sterile adsorbent paper, cut into 0.5 cm long sections using sterile blades and plated on 9 cm Petri dishes filled with Nutrient Yeast Dextrose Agar (NYDA) medium containing 0.015% cycloheximide and incubated at 26°C for 24 h.

Isolation of endophytic bacteria was performed by surface sterilizing wheat stems in 5% NaClO for 1 min and then rinsing in sterile distilled water. Stem sections of approximately 15

cm above the crown were kept for sap extraction. Wheat tissue sap was extracted using the new method, the CIHEAM-IAMB patented 'Method for the extraction of sap from plant material and apparatus for carrying out the method' (https://patents.google.com/patent/WO2017017555A1/en). Wheat sap extract was diluted 1:10 in sterile PBS solution until reaching 10^{-2} and 100 µl of both diluted and not diluted sap solutions were plated on 9 cm Petri's dishes filled with NYDA added with 0.015% cycloheximide and incubated at 26°C for 24 h.

Grown bacteria from all three matrices were purified by transferring them into fresh non-selective NYDA and then King's B media in order to obtain pure single colonies to be screened for PGP activities. At each transfer, inoculated plates were incubated at 26°C for 24-48 h.

Preliminary tests and screenings for plant growth promoting activities

Culture-isolated bacteria were tested using potassium hydroxide (KOH) in order to evaluate bacterial wall type by stirring a bacterial colony in a drop of 3% v/v KOH (Gregersen, 1978). Positive reaction to KOH was visible in presence of gram-negative bacteria producing an increase of liquid viscosity; gram positives, instead, produced a watery suspension. Gram positive bacteria were also tested for spore production by growing the bacterial colonies in 7 ml of Nutrient Broth (Sigma Aldrich, Italy) at 27°C for 24 h at 200 rpm. Afterwards, 1 ml of the bacterial suspension was transferred into a sterile 1.5 ml tube and pasteurized at 80°C for 15 min. After the incubation, 100 µl of the pasteurized and non-pasteurized bacterial suspensions were plated on King's B Agar and incubated at 27°C for 24-48h. The non-pasteurized cultures were used as control to check the vitality of the tested bacteria, whereas growth in plate culture of the pasteurized cultures was interpreted as positive result to spore production (Frank *et al.*, 2004).

Furthermore, Triple Sugar Iron Agar (TSI; Sigma Aldrich) was used to identify potential human pathogenic Enterobacteriaceae that were removed from further tests. The remaining bacterial colonies were progressively screened for PGP activities by keeping colonies able to perform at least more than two activities. The PGP activities tested were: fluorescence production, inorganic phosphate (Pi) solubilization, ammonium (NH_4^+), indoleacetic acid (IAA) and siderophores production (Bric *et al.*, 1991; Ahmad *et al.*, 2008; Kumar *et al.*, 2012).

- *Fluorescence production*

Fluorescence was evaluated on gram negative bacteria by spreading single colonies on King's B Agar medium prepared as follows: Pseudomonas Agar F (Merk) 35 g/L, Glycerol 12.6 g/L, Agar 3 g/L. Inoculated plates were incubated at 26˚C for 24-48h and used for visualisation of fluorescence performed by placing the plates with the grown colonies under UV light.

- *Pi solubilization*

Solubilisation of inorganic phosphate was performed by marking six sections on a Petri's dish containing National Botanical Research Institute Phosphate medium (NBRIP), Glucose 10 g/L; $Ca_3(PO_4)_2$ 5 g/L; $MgCl_2 \cdot 6H_2O$ 5 g/L; $MgSO_4 \cdot 7H_2O$ 0.25 g/L; KCl 0.2 g/L; $(NH_4)_2SO_4$ 0.2 g/L; Agar technical 15 g/L. Single bacterial colonies were spotted within each section three times in order to allow a easier evaluation of the result and using a sterile loop. Plates were incubated at 26˚C for 48h and development of a clear halo around the bacteria was interpreted as positive result.

- *NH_4^+ production*

Single colonies were inoculated in 15 ml culture tubes filled with 7 ml of sterile buffered peptone water (Sigma Aldrich, Italy) and incubated at 26˚C for 24h. Evaluation of ammonium production was performed by adding 200 µlof Nessler reagent. Change of colour from milky white to yellow/brown and the presence of precipitate was the indicator of NH_4^+ production.

- *IAA production*

Six sections were marked on each Petri's dishes containing modified Luria-Bertani Agar (Bacto-triptone 10 g/L; Yeast extract 5 g/L; NaCl 10 g/L; Bacto-agar 15 g/L; L-Triptophan 5 mM) using a marker and three single colonies of the same bacterial strain were transferred into each section in order to test six bacterial strains in each plate in triplicate. In addition, squared sections of sterile filter paper were placed on each bacterial inoculum and inoculated plates were incubated at 26˚C for 48 h.

A layer of adsorbent paper was placed into clean Petri's dishes and wet with Salkowsky's reagent A (2% FeCl 0.5M in $HClO_4$ 35%). Therefore, the sections of filter papers placed on top of the grown bacteria were transferred on the adsorbent paper wet with the Salkowsky's reagent A using clean tweezers. A pink halo was observed on the filter papers belonging to IAA producing colonies after 15 min of incubation in the dark and at room temperature.

- *Siderophores production*

Bacterial colonies were spotted on King's B Agar using a sterile toothpick and incubated at 26˚C for 48h. Overlay Chrome Azurol S medium (O-CAS) was prepared according to Nautiyal (1999) modified by Pérez-Miranda and co-workers (2007). Briefly, a colouring solution was prepared by mixing a solution of 60.5 mg Chrome Azurol S (CAS) in 50 ml H_2O, 10 mL of a solution 1 mM FeCl3·6H2O in 10 mM HCl and a solution of 72.9 mg of Hexadecyltrimethylammonium bromide (HDTMA or CTAB) in 40 ml of H_2O. In addition, a Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES) solution was prepared by solubilizing 30.24 g PIPES and 9 g of Agarose in 750 ml H_2O; pH was adjusted to 6.8 with a solution of 50% (w/w) NaOH. Colouring and PIPES solutions were then mixed together and autoclaved at 121˚C for 20 min. Once cooled until reaching a temperature below 40°C a layer of O-CAS medium was poured into Petri's dishes with the grown bacteria until the colonies were completely covered. An orange/yellow halo was visible around the siderophores producing colonies after 15 min of incubation at room temperature.

Data analyses

The frequencies of the bacterial types (gram positives and gram negatives) and the PGP activities (phosphorus solubilization and fluorescence, ammonium, indoleacetic acid and siderophores production) performed by the bacterial colonies belonging to the two fields (Field.1 and Fied.2) and the three matrices under study (bulk-soil:November 2015; wheat roots: DoY 73; wheat sap: DoY131) were analyzed and compared. In addition, the average of spatial variability of the PGP activities related to the single sampling area within each field was analyzed.

A Discriminant Analysis (DA) was conducted to evaluate the significance of the variability observed among the average of the detected PGP activities (independent variables) by setting the matrix and the field as "group variables".

The homogeneity of the covariance matrices inside the groups was tested using the M-Box test. The standardized coefficients related to the discriminant variables extracted that were significant to the Willks' Lambda test were considered for the interpretation of the results.

Finally, a Multivariate Linear Regression analysis was carried out using a General Regression Model (GRM) to evaluate the relationships of the average of plant height (APH, morphometric variable) and the HVIs related to wheat growth Slope, Vi_{opt}, PSSRa and PSSRcar (see Chapter 2) with the PGP activities (predictors) detected from the screened

bacterial colonies. The model RM-GRM was run using the option "best subset" which allowed to select the most significant PGP activities based on the coefficient of determination, R^2 and Mallow's Cp (Mallows, 1995), which objectively esteems the Mean Square Prediction Error (MSPE).

All the statistical analyses were carried out using Statistica v.8 run in a Windows environment (StatSoft inc.).

Results

Isolation and screening for PGP activities of soil borne and wheat-associated bacteria

The culture-dependent approach allowed to isolate 82 bacteria from bulk soil, 157 from wheat roots and 129 from wheat sap for an overall of 368 bacterial colonies.

Among the isolated bacteria 36, 14 and 17 were gram positive, whereas 46, 143 and 112 were gram negative in soil-borne, root-associated and endophytic bacteria respectively.

After the preliminary screening using TSI test, 102 colonies were excluded from the three matrices as their pattern matched with potential human pathogenic *Enterobacteriaceae*. Therefore, 51 soil-borne, 137 root-associated and 60 endophytic colonies were further screened for PGP activities *in vitro* as shown in Figure 18.

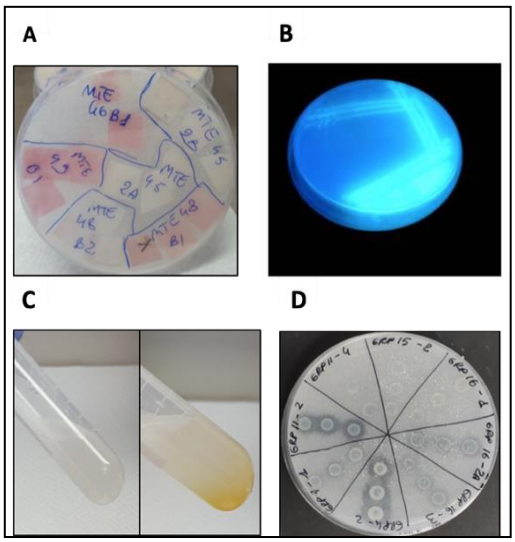

Figure 18. Microbial screenings for plant-growth promoting activities. *Examples of screening for indoleacetic acid (A), fluorescence under UV light (B), ammonium production (C) and solubilization of inorganic phosphorus (D) performed on bacterial colonies isolated from soil and wheat plant material.*

Most of the bacteria isolated from bulk soil were gram-positive whereas gram-negatives were predominant among wheat-associated isolates (Figure 19). Furthermore, microbial screenings allowed to detect several colonies performing PGP activities.

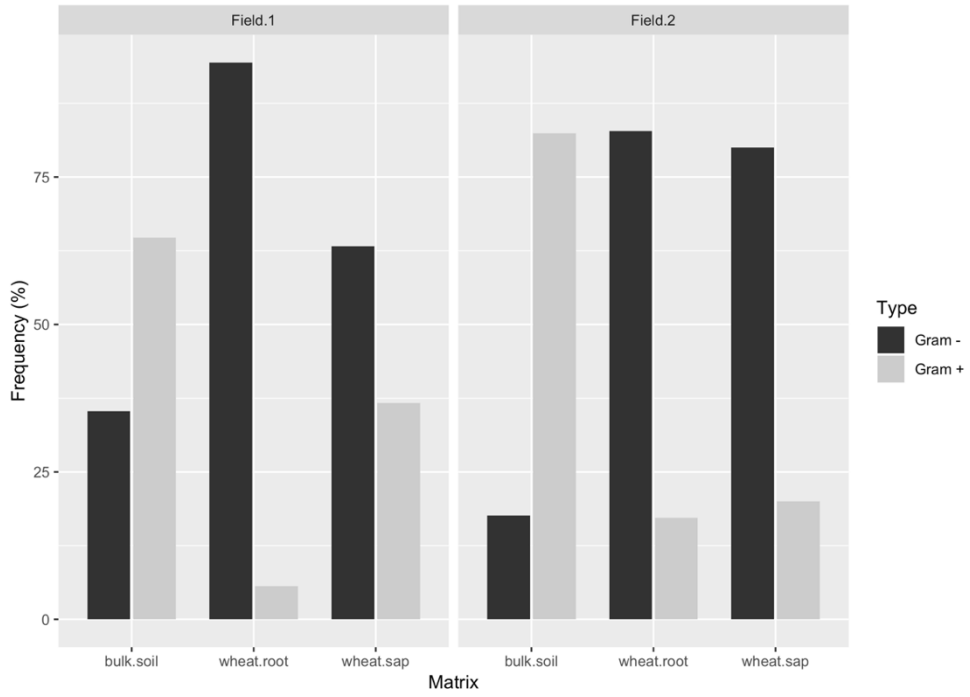

Figure 19. Frequencies of bacteria isolated from three matrices. *The graph shows the proportions in terms of gram-positive (gram +) and gram-negative (gram -) bacteria isolated from the two fields and the three matrices under study: bulk soil, wheat roots and wheat sap (endophytic).*

The proportions of the detected PGP activities performed by bacterial colonies isolated from wheat in the two fields are shown in Figure 20.

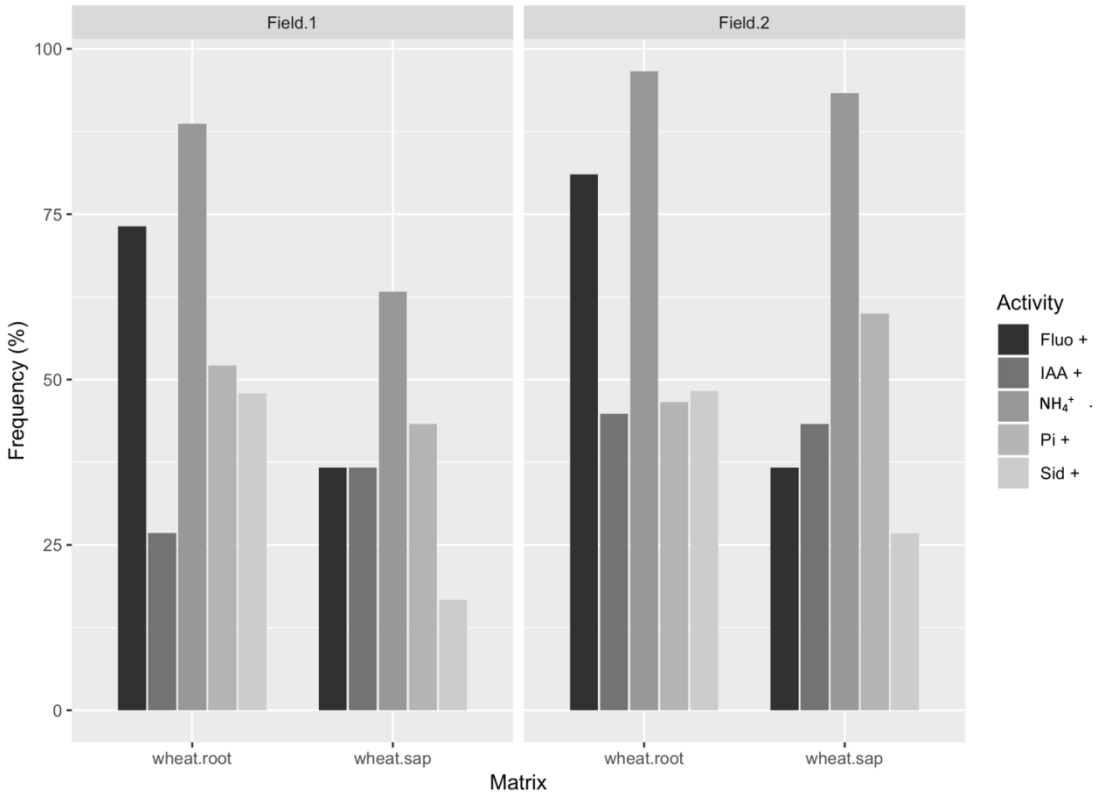

Figure 20. Plant-growth promoting activities detected in wheat tissues. *Proportions of PGP activities detected in bacterial colonies isolated from wheat roots and wheat sap from the two fields under study. Fluo +: fluorescent gram negatives; IAA +: indoleacetic acid production; NH_4^+: ammonium production; Pi+: solubilization of inorganic phosphorus; Sid+: siderophores production.*

After TSI test, 34 and 17 colonies isolated from bulk soil and belonging respectively to field 1 and field 2 were screened. In terms of PGP activities, a lower percentage of fluorescence producing bacteria were isolated from field 2 compared to field 1.

Bacterial colonies isolated from wheat roots surface accounted 71 and 58 colonies belonging to field 1 and field 2 respectively. The PGP activities performed by the screened bacteria showed similar proportions between the two fields, except for indoleacetic acid (IAA) producing colonies, whose percentage in field 2 resulted higher compared to field 1.

The endophytic bacteria isolated from wheat tissue sap that were screened for PGP activities after the TSI test included 30 colonies per field. Overall, similar proportions of PGP activities were observed between the colonies isolated from the two fields.

Among the screened isolates 11 soil-borne, 48 root-associated and 18 endophytic bacterial colonies showed positivity to multiple PGP activities. Among them, 24 isolates were selected

for further testing, listed in Table X, in order to include as much diversity as possible in terms of activities performed and colony morphology.

Table X. Potential PGPR strains selected based on "in vitro" screenings. *Bacterial strains showing positivity to multiple PGP activities selected from the three matrices under study to be further analyzed and tested.* ***Fluo:*** *fluorescence;* ***NH_4^+:*** *ammonium production;* ***Pi:*** *solubilization of inorganic phosphorus;* ***IAA:*** *indoleacetic acid production; Sid: siderophores production.* ***Np.:*** *not performed;* ***Na.:*** *not available.*

ID	Gram	Spore	Fluo	NH_4^+	Pi	IAA	Sid
ENDOPHYTES							
8	-	np.	-	+	++	++++	+
15	+	+	np.	+++	-	-	+
20	+	-	np.	-	-	+++	-
35	-	np.	+	++	++	-	+
39	-	np.	+	++	++++	-	+
49	-	np.	+	++	++	-	+
63	+	-	np.	++++	-	+	-
84	+	-	np.	na	++	-	-
91	-	np.	+	+	++	++	-
92	-	np.	+	+	+	+++	-
101	-	np.	+	+++	+	-	+
130	-	np.	+	+++	+++	-	+
ROOTS							
41	-	np.	+	++	+	++	+
85	-	np.	+	+++	+++	-	+
86	-	np.	+	+	+	++	+
126	-	np.	-	++	++	++	+
138	-	np.	+	+	-	+++	+
159	-	np.	+	++++	++	+	+
179	-	np.	+	+++	+++	+	+
186	-	np.	+	+++	++	++	-
BULK SOIL							
1	+	-	np	-	+	-	na
3	-	np	+	+	+	-	+
5	+	+	np	-	+	-	na
7	-	np	+	+	+	-	-

Variability of the detected PGP activities between the three matrices and the two fields

The main descriptive statistics for the detected PGP activities showed almost multinormal distributions, except for fluorescence production (Table XI).

Table XI. Descriptive statistics of the data related to the PGP activities detected from the screened bacterial colonies isolated from wheat tissues.

PGP activities	Valid N	Mean	Minimum	Maximum	Variance	Skewness	Kurtosis
NH_4^++	96	1.312500	0	4	1.248684	0.553636	-0.567577
Pi+	96	2.072917	0	6	1.541996	0.667642	0.239869
IAA+	96	1.062500	0	4	0.943421	0.788041	0.368622
Fluo+	96	0.812500	0	4	0.932895	1.318171	1.541691
Sid+	96	0.781250	0	4	0.762171	1.026270	0.909468

The M-Box tests performed on the groups “Matrix” and “Field” showed contrasting results. Indeed, the PGP activities (levels) grouped by "Matrix" showed highly significant covariance separation (p-level < 0.05), whereas the levels of the "Field" group were more homogeneous (p-level > 0.05) (Table XII).

Table XII. Results of the M-Box tests performed on the PGP activities grouped by Matrix and by Field.

Group	PGP activities*	Box-M	Chi-sqr	df	p-value
Matrix	NH_4^++	54.09	48.12	12	0.000003
	Pi+				
	IAA+				
Field	NH_4^++	15.97	15.06	15	0.447
	Pi+				
	IAA+				
	Fluo+				
	Sid+				

*PGP activities showing non-zero variance within the group.

These results, along with the multinormal distribution observed from the values of Skewness and Kurtosis associated to the PGP activities (almost close to zero; Table I) fulfilled the conditions for performing the discriminant analysis (DA) on the "Matrix" group. Results from DA allowed to extract two highly significant discriminant components (DCs) from the "Matrix" group corresponding to Wilks' Lambda values close to zero (Table XIII) graphically showed in Figure 21.

Table XIII. Results obtained from the discriminant analysis performed on the PGP activities grouped by "Matrix".

Group	DCs	Eigenvalue	% of variance ratio explained	Wilks' Lambda	p-level
Matrix	1	0.641	74,84	0.50	0.000000
	2	0.215	25.16	0.82	0.001377

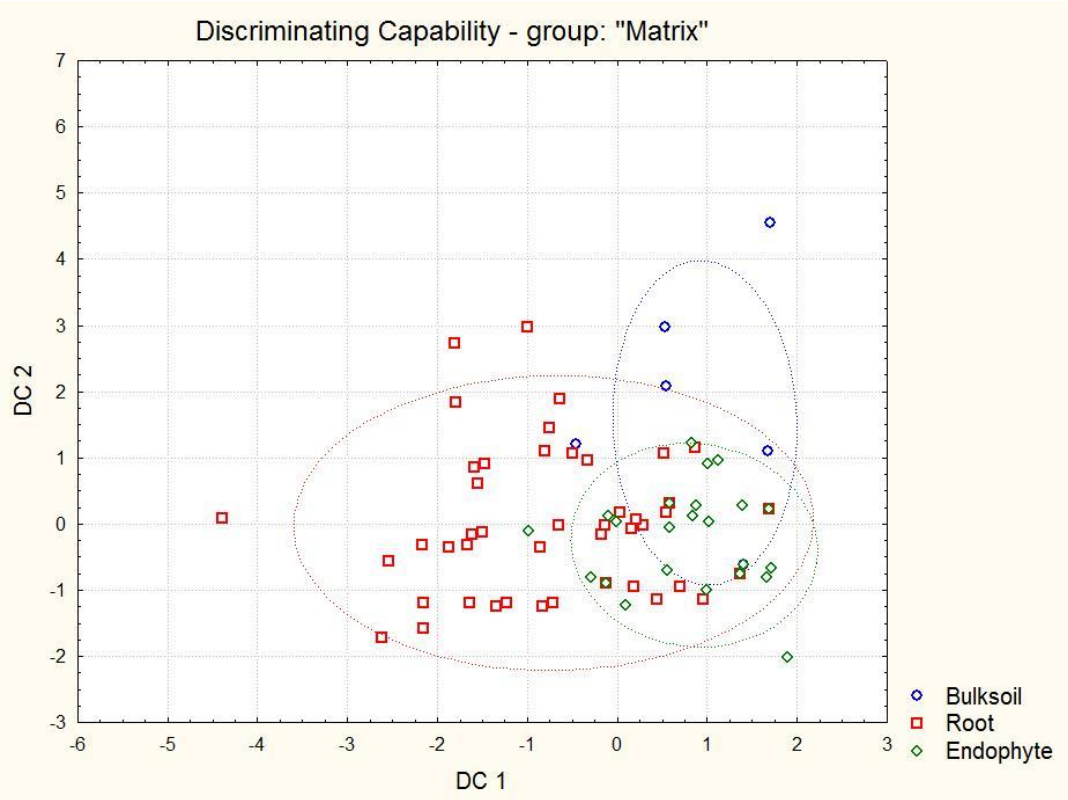

Figure 21. Discriminant analysis on "Matrix" group. *Scatterplot showing the separation of the PGP activities performed by bacterial colonies belonging to the three matrices (levels) under study: bulk-soil, wheat roots and wheat sap (endophytes) based on the two discriminant components (DC1 and DC2) extracted from the "Matrix" group.*

Discriminant components showed a separation between the levels of the "Matrix" group, Bulk-soil, Root and Endophyte in relation to DC 1 (Bulksoil & Endophyte vs. Root) and with respect to DC 2 (Bulksoil vs. Root & Endophyte).

This separation was significantly quantified by the *distance of Mahalanobis* measured between the centroids of the three matrices (Table XIV).

Table XIV. Squared Mahalanobis Distances. *F-values and p-levels to quantify the separation between the levels in the "Matrix" group.*

	Bulksoil [F; p]	Root [F; p]	Endophyte [F; p]
Bulksoil [F; p]	**0**	**5.12** *[6.06; 0.000071]*	**3.43** *[3.84; 0.003353]*
Root [F; p]	**5.12** *[6.06; 0.000071]*	**0**	**2.56** [10.5; *0.000000*]
Sap [F; p]	**3.43** *[3.84; 0.003353]*	**2.56** [10.5; *0.000000*]	**0**

The analysis of the *standardized coefficients* allowed to identify the PGP activities that maximize the discrimination within the "Matrix" group (Figure 21). In addition, DC 1 expressed the 74,84 % of the total variance, whereas DC 2 explained the remaining 25,16 % (Table XV).

Based on DC1, NH_4^+ and, to a lesser extent, siderophores production activities were the main responsible for the average spatial variability of the PGP activities between the matrices, Root and Bulksoil/Sap (negatively correlated). Moreover, DC2 showed a direct contribution of phosphorous solubilization and and inverse contribution of indoleacetic acid production to the differences between Bulksoil and wheat Sap (Table XV).

Table XV. Total-sample Standardized Coefficients and Correlations Variables for DC 1 and DC 2. NH_4^++: *ammonium production**; Pi+:** phosphorus solubilization**; IAA+:** indoleacetic acid production**; Fluo**+: fluorescence production**; Sid+:** siderophores production.*

	PGP activity	DC 1	DC 2
Group: "Matrix"	**NH_4^++**	**-0.759922**	0.043024
	Pi+	-0.019206	**1.011981**
	IAA+	-0.023113	**-0.929432**
	Fluo+	0.291042	-0.046785
	Sid+	**-0.512362**	-0.148502
	Cum. Prop.	0.748438	1.000000

Regarding the PGP activities exclusively related to plant growth (extraction from wheat roots and wheat sap at Doy 73 and 131), NH_4^+ and siderophores production were the two main activities determining the spatial differentiation.

Finally, the discriminatory analysis performed on the "Field" group (field.1 and field.2) did not show any evident spatial variability between the PGP activities. The growth mechanisms in the two fields proceeded in the same way.

Analysis of the relationships between bacterial plant-growth promoting activities and hyperspectral vegetation indices associated with wheat growth

The correlations between PGP activities, APH and HVIs were studied by conducting five Multivariate Linear Regression analyses. In all cases, the partial regression coefficients (B) of APH, Slope, Vi_{opt}, PSSRa and PSSRcar were statistically different from zero with very significant t-Student values (Table XVI).

Table XVI. Multivariate Linear Regression. *Results of the analyses performed on the selected HVIs related to wheat growth Slope, Vi_{opt}, PSSRa and PSSRcar and the average of plant height (APH) derived from morphometric measures taken on the two ancient wheat landraces "Perciasacchi" and "Tumminia".*

Dependent Variable:	**Multiple R**	**Multiple R^2**	**Adjusted R^2**	**Cp**	**F**	**p-level**
APH	0.432	0.186	0.175	0.632	16.72	0.000110
Variables in the Equation	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			67.073	4.494	14.92	0.000000
NH4+	**-0.432**	**0.106**	**-9.833**	**2.404**	**-4.09**	**0.000110**
Pi+	-	-	-	-	-	-
IAA+	-	-	-	-	-	-
Fluo+	-	-	-	-	-	-
Sid+	-	-	-	-	-	-

Model: **APH** = 67.073 - 9.833 * **NH4+**

Dependent Variable:	**Multiple R**	**Multiple R^2**	**Adjusted R^2**	**Cp**	**F**	**p-level**
Slope	0.397	0.158	0.135	1.841	6.93	0.001747
Variables in the Equation	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			-0.032	0.0021	-15.27	0.000000
NH4+	**0.251**	**0.121**	**0.0025**	**0.0012**	**2.07**	**0.041682**
Pi+	-	-	-	-	-	-
IAA+	-	-	-	-	-	-
Fluo+	-	-	-	-	-	-
Sid+	0.210	0.121	0.0027	0.0015	1.73	0.087383

Model: **Slope** = -0.032 + 0.0025 * **NH4+** + 0.0027 * **Sid+**

Dependent Variable:	**Multiple R**	**Multiple R^2**	**Adjusted R^2**	**Cp**	**F**	**p-level**
Vi_{opt}	0.350	0.123	0.111	-0.669	10.50	0.001778
Variables in the Equation	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			3.158	0.013	239.80	0.000000
NH4+	**-0.350**	**0.108**	**-0.023**	**0.007**	**-3.24**	**0.001778**
Pi+	-	-	-	-	-	-
IAA+	-	-	-	-	-	-
Fluo+	-	-	-	-	-	-
Sid+	-	-	-	-	-	-

Model: **Vi_{opt}** = 3.158 - 0.023 * **NH4+**

Dependent Variable:	Multiple R	Multiple R^2	Adjusted R^2	Cp	F	p-level
PSSRa	0.256	0.066	0.053	-0.784	5.28	0.024373
Variables in the Equation	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			4.779	0.072	66.51	0.000000
NH4+	**-0.256**	**0.112**	**-0.089**	**0.039**	**-2.30**	**0.024373**
Pi+	-	-	-	-	-	-
IAA+	-	-	-	-	-	-
Fluo+	-	-	-	-	-	-
Sid+	-	-	-	-	-	-

Model: **PSSRa** = 4.779 - 0.089 * **NH4+**

Dependent Variable:	Multiple R	Multiple R^2	Adjusted R^2	Cp	F	p-level
PSSRcar	0.296	0.088	0.076	0.003	7.23	0.008825
Variables in the Equation	**Beta**	**Std.Err. of Beta**	**B**	**Std.Err. of B**	**t**	**p-level**
Intercept			4.641	0.075	61.42	0.000000
NH4+	**-0.296**	**0.110**	**-0.110**	**0.041**	**-2.69**	**0.008825**
Pi+	-	-	-	-	-	-
IAA+	-	-	-	-	-	-
Fluo+	-	-	-	-	-	-
Sid+	-	-	-	-	-	-

Model: **PSSRcar** = 4.641 - 0.110 * **NH4+**

The linear equations showed low F values, indicating low adequacy of the models to explain the variability of the data, whereas the correlations were highly significant, especially for APH and Vi_{opt}.

Moreover, all the estimated variables showed low values of the multiple determination coefficient R^2. However, the best estimate was observed for APH and the two growth indices Vi_{opt} and Slope that were linearly correlated with NH_4^+ production (directly to Slope and inversely to APH and Vi_{opt}). In this case, siderophores production (Sid+) showed a trend of direct relationship with the Slope index, although the p-value was not statistically significant.

The analysis of residuals was carried out for the estimation of Vi_{opt} compared to the predictor NH_4^+ production which was the best model obtained. The residual distributions of Vi_{opt} data was close to normality (Figure 22A) as confirmed by the Normal Probability Plot of the residuals (Figure 22 B) and the Partial Residual Plot which is related to NH_4^+ production (Figure 22 C) showing a linear dependence.

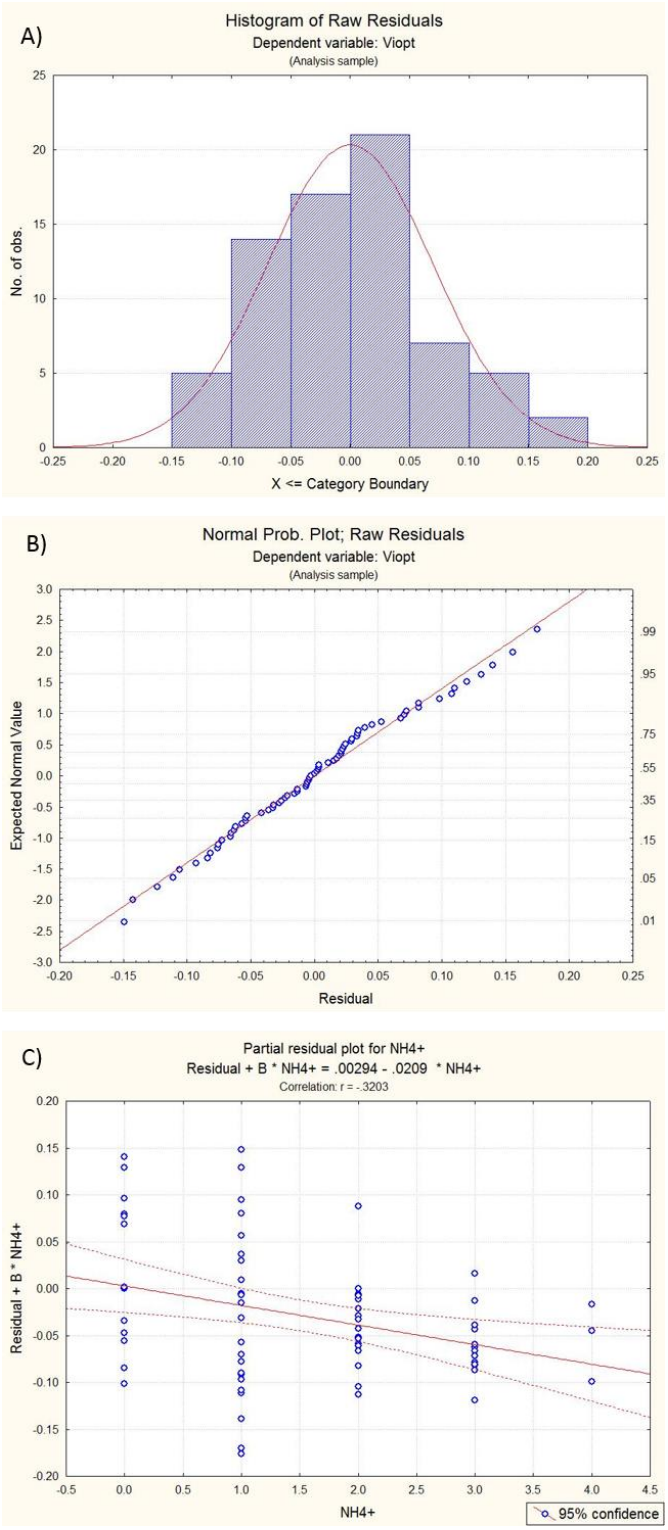

Figure 22. Histogram of the residual distribution *related to the Viopt (A), Normal Probability Plot of the residuals showing the points almost completely lying on the line which confirms the normality of residual distribution (B) and the Partial Residual Plot related to NH4+ production (C).*

Discussion

This study allowed to detect the presence of beneficial bacteria associated to two ancient tetraploid wheat landraces native of the Sicilian territory, Perciasacchi and Tumminia. Moreover, some of the tested bacteria showed positivity to multiple PGP activities *in vitro* conditions.

Despite the differences in the agricultural practices no significant differences in bacterial types (based on gram classification) or activities was found between the two fields. Probably, the effects of crop rotation on the soil bacterial composition cannot be properly observed by considering only the gram classification. However, differences in the proportions of gram-positive and gram-negative bacteria were observed between bulk soil and wheat tissues. These results are coherent with the role of the host genotype in recruiting plant-associated microorganisms from the environment shaping the plant-associated microbial community (Sapkota *et al.*, 2015).

Furthermore, the laboratory conditions provided a simplified environment to evaluate the ability of bacterial colonies isolated in pure culture to perform beneficial activities for plant growth. However, the behaviour of the studied bacteria as well as their ability of performing the detected activities might be affected by the interactions with the host and the surrounding environment once they are applied in the open field (Lambert and Joos, 1989). Indeed, to be effective in enhancing plant growth, *in vitro* selected bacteria have to be able to successfully establish within the plant-associated microbial community and tissues as well as being able to perform their activities within the microbes-crop-environment combination they are applied to (Martínez-Viveros *et al.*, 2010). For this reason, the selected bacteria need to be further tested in order to evaluate their effects *in vivo* on wheat growth. In addition, it might be interesting to investigate the possibility of transferring beneficial bacteria associated to ancient wheat landraces to modern wheat varieties as well as combination of isolates in order to enhance their efficiency.

Therefore, a further investigation about the effects of the selected bacteria on wheat growth has been performed in glasshouse conditions, by taking into accounts modern and ancient wheat varieties as described in chapter 4.

In addition, differences in the frequencies of PGP activities were observed among the three matrices and this trend was coherent with the results from statistical analyses. The separation between the three matrices was not immediately observed by the graph, probably due to

deviations from the multinormality of the variables and to the heterogeneity of Variance-Covariance of the groups (Tabachnick and Fidell, 2001). However, the distances from the centroids confirmed the separation of the groups and the values of Skewness and Kurtosis close to zero allowed to proceed with the multivariate analysis of the group "Matrix".

Finally, interesting correlations were observed between the selected HVIs (see Chapter 2) and some of the bacterial PGP activities detected in this study. Despite the low values obtained from the multiple determination coefficient R^2, NH_4^+ production the correlation with Slope, Vi_{opt}, PSSRa and PSSRcar was strongly significant. In addition, the linear correlation between NH_4^+ production from wheat-associated bacteria and Vi_{opt} is coherent with the physiological parameter of leaf nitrogen content associated with this HVI (Reyniers *et al.*, 2006) and the relationships previously reported between nitrogen and pigment contents in plant leaves (Ercoli *et al.*, 1993). In conclusion, the results obtained in this study suggest the possibility of using proximal sensing to estimate growth conditions related to bacterial PGP activities in the field. Moreover, the study of a broader spectrum of HVIs and of more regions of the hyperspectral signatures might allow to identify more correlations between HVIs and the plant-associated microbial activities providing helpful information for a more efficient isolation of beneficial strains to be used for improving agriculture sustainability.

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Chapter 5

Effects of Plant-Growth Promoting Rhizobacteria (PGPR) on wheat growth and susceptibility to insect pests

Abstract

Aphid infestations are responsible for significant yield losses in many important crops causing both direct damage to the plant and by transmitting plant viruses. In particular, *Rhopalosiphumpadi*and *Sitobionavenae*are the most studied cereal pests carrying multiple plant viruses and inhibiting plant growth in several cereal crops, including wheat. Numerous studies have shown that plant growth promoting rhizobacteria (PGPR) can reduce insect pest fitness by eliciting plant systemic defences and release of volatile compounds to attract natural enemies. Considering the importance of tetraploid wheat in Italy and the increasing interest in the ancient wheat landraces as a source of plant-associated microbial biodiversity in recent years, this study aimed to evaluate the effects of candidate PGPRs isolated from two ancient wheat landraces, Perciasacchi and Tumminia, grown in Sicily. Evaluation of plant performance in terms of plant growth and susceptibility to aphid pests (*R. padi* and *S. avenae*) were evaluated using a modern (Cordiale) and an ancient (Perciasacchi) wheat cultivar. Plant responses to treatments and aphid infestations were strongly related to the host genotype. In addition, a mixture of autoclaved bacteria positively affected plant growth in Cordiale plant and the bacterium *Pseudomonas clororaphis* showed significant positive effects on plant growth in Perciasacchi plants.

Introduction

Insect pests, including aphids, are responsible for significant yield losses in many important crops causing both direct damage to the plant and by transmitting plant viruses (Parizoto *et al.,* 2013; Parry, 2013). In particular, *Rhopalosiphum padi* and *Sitobion avenae* are the most studied cereal pests carrying multiple plant viruses and inhibiting plant growth in several cereal crops, including wheat (Greenslade *et al.,* 2016; Foster *et al.,* 2014).

Aphid infestations can develop very quickly during spring and summer due to an asexual stage (anholocyclic) in their life cycle (Dedryver *et al.,* 2001; Blackman and Eastop, 2000). Asexual generations are produced in most aphid species through parthenogenesis, when secondary hosts are readily available in the summer. A sexual life stage occurs in autumn when the days become shorter and the temperature falls and depends on the presence of the primary host. However, in milder climates, some species can survive through winter without a sexual phase (Pons *et al.,* 1993; Leather, 1992). Wingless aphids develop on the flag and upper leaves of cereals and grasses. As the plant matures, aphids move to the developing flowers (the emerging ears in the case of wheat). Winged forms develop in late May and throughout the summer in response to increased population density and crowding and decline of food plant quality and availability for establishing new colonies. In autumn they move to re-infest cereals or other grasses

(http://influentialpoints.com/Gallery/Sitobion_avenae_English_Grain_aphid.htm).

Presently, the principal method adopted to reduce crop damage caused by aphids is insecticide applications. However, this strategy does not support long-term sustainability due to the negative environmental impact of pesticides on non-target organisms and the risk of developing insecticide resistance in pest populations (Devonshire and Field, 1991; Smith and Boyko, 2007). Consequently, alternative pest control methods have been adopted in order to reduce chemical applications such as biological control using natural enemies of crop pests (Ramsden *et al.,* 2016). Furthermore, studies aimed at identifying pest resistance and tolerance traits to be used in crop breeding programmes have been carried out with encouraging results (Girvin *et al.,* 2017; Mitchell *et al.,* 2016), often by studying wild relatives of modern cultivars (Delp *et al.,* 2009).

Recently, the study of plant-associated microbial communities, known as plant microbiota, has raised particular interest in their ability to influence plant growth, physiology and response to herbivorous pests (Bender *et al.,* 2016; Berendsen *et al.,* 2012). Furthermore,

there is evidence that beneficial microorganisms, including plant growth promoting rhizobacteria (PGPR), can reduce insect pest fitness by eliciting plant systemic defences and release of volatile compounds to attract natural enemies (Kessler and Heil, 2011). Consequently, several studies have been conducted to identify microbial strains that can influence plant responses to insect pests (reviewed in Pineda *et al.,* 2015; Pineda*et al.,* 2010; Rasmann *et al.,* 2017). Furthermore, encouraging results were obtained by inoculating wheat plants with strains of *Bacillus* sp. and *Pseudomonas* sp. to reduce aphid fitness (Nawaz and Hussain, 2018).

These promising results led to a further investigation of the wheat-associated microbial community by studying ancient wheat landraces (*Triticum durum* and *T. turgidum*) that are acquiring great interest in the south of Italy in recent years. In particular, in Sicily, the flours of two ancient wheat landraces, Perciasacchi and Tumminia, are considered highly valuable for their peculiar organoleptic properties (Vita *et al.,* 2016). Moreover, the interest in ancient landraces is growing for their low fertilization requirements and ability to grow in sub-optimal environmental conditions (Pecetti *et al.,* 1994).Considering that host genotype is reported to play a role in shaping the composition of microbial diversity associated with plant roots and tissues, there might be a possibility of finding beneficial microorganisms associated with ancient landraces and contributing to their ability to perform well in low-fertility and dry soils. Furthermore, these microorganisms might be transferable to modern wheat cultivars to improve their growth and health.

In this framework, the study of PGPRs could lead to the application of beneficial bacteria in the field in order to improve plant growth and agriculture sustainability. Therefore, the aim of this work was to evaluate the effects of a set of candidates PGPR strains isolated from two ancient tetraploid wheat varieties (Perciasacchi and Tumminia) native to the Sicilian territory, and selected based on their *in vitro* activities, on the performance of a modern and an ancient wheat cultivar. Plant performance was evaluated in terms of plant growth and susceptibility to aphid pests.

Materials and methods

Taxonomic identification of bacterial strains

Bacterial inoculants to be evaluated were isolated from bulk-soil, root surface and tissue sap (Yaseen, 2015) of two Italian tetraploid wheat landraces native to the Sicilian territory, Perciasacchi (winter wheat) and Tumminia (spring wheat). *In vitro* culture-based screenings for plant beneficial activities were performed as described in chapter 3 in order to select the most active strains.

Twenty-five bacterial isolates showing ability of performing more than two PGPR activities *in vitro*, were identified taxonomically through Sanger sequencing of the 16S rRNA gene. DNA was extracted from single colonies using the "Nucleospin Tissue" Kit (Macherey-Nagel, Germany) and DNA quality and quantity was checked using a NanoDrop® ND-1000 Full spectrum UV-Visible Spectrophotometer (ThermoFischer Scientific, Epsom, UK). A fragment of 1500 bp of the 16S rRNA was amplified by Polymerase Chain Reaction (PCR) using primers 27F (5' - GAGTTTGATCMTGGCTCAG - 3') and 1392R (5' - ACGGGCGGTGTGTRC -3') (Lane, 1991). Amplification of the target sequence was performed in 20 µl reactions containing5X "KAPA HiFi Buffer" (KAPA Biosystems, USA) reaching a final concentration of 1X, comprising BSA 0.05 ng/µl, 0.3 mM KAPA dNTP mix (KAPA Biosystems, USA), 0.3 µM of each primer and 0.3 U of 1 U/µl KAPA (KAPA Biosystems, USA). A volume of 5 µl of target DNA (approximately 15-20 ng/ul) was added to the reactions and amplification was carried out under the following conditions: 94°C for 3 min followed by 25 cycles at 98°C for 30 s, 58°C for 30 s and 72 for 1.5 min and a final elongation step at 72°C for 10 min. PCR products were visualized by electrophoresis in 1.5% agarose gel stained with SYBR Safe®. Single band amplicons were purified using PCR clean-up kit (Qiagen, UK) following the manufacturer protocol. Multiple band PCR products were run on 1.5% agarose gel stained with SYBR Safe® and the 1500 bp fragment was cut and purified using QIAquick Gel Extraction Kit (Qiagen, UK). Purified PCR products were quantified using the Nanodrop ND-1000 (ThermoFischer Scientific, UK) and samples reaching DNA concentrations of at least 10 ng/µl were sequenced using Sanger methodology using a 36cm capillary array on a 48 capillary ABI 3730 (Life Technologies Ltd, Paisley, UK) at the sequencing facility at The James Hutton Institute (Invergowrie, UK).

Raw sequences were analysed using Chromas v. 2.6.5 and taxonomic identification was conducted by alignment of the obtained sequences on NCBI BLAST database (Altschul *et al.*,

1997; Altschul*et al.*, 1990). Finally, potential human and plant pathogens were excluded from the identified strains using scientific references and online official databases such as "Catalogue of microorganisms" on DSMZ database (https://www.dsmz.de), which is based on the German classification: 466 Classification of prokaryotes (bacteria and archaea) into risk groups (https://www.baua.de/EN/Service/Legislative-texts-and-technical-rules/Rules/TRBA/TRBA-466.html).

Greenhouse experiments

Two experiments were conducted in greenhouse conditions using two winter wheat varieties: Perciasacchi, the Italian ancient variety from which some of the tested strains were isolated; and Cordiale, a modern UK variety chosen from the wheat collection of the James Hutton Institute.

A randomized block design comprising a total of 280 plants distributed between five blocks, each block containing two replicates (ten replicates in total) of each of 14 treatments and wheat varieties, was used to account for the effect of plant position in the glasshouse cubicle on the measures.

The first experiment consisted of the inoculation of wheat plants with 12 identified isolates to identify the strains most effective at enhancing wheat growth. The strain used in this experiment, here referred to as "treatments", were: *Brevibacterium frigoritolerans* (T01), *Pseudoarthrobacter siccitolerans* (T02), *Pseudomonas chlororaphis* (T03), *Stenotrophomonas chelatiphaga* (T04), *Pseudomonas cedrina* (2 strains: T05 and T06), *Pseudomonas extremorientalis* (T07), *Pseudomonas mucidolens* (T08), *Pseudomonas helmanticensis* (T09), *Pseudomonas koreensis* (T10), *Microbacterium maritypicum* (T11) and *Pseudomonas vancouverensis* (T12). In addition, two groups of control plants were inoculated with autoclaved mixed bacterial inoculum (containing an equal concentration of each inoculum: Ctrl1) and with sterile distilled water (Ctrl2).

The second experiment was conducted using treatments T01, T03, T05 and T07 from the first experiment in order to evaluate their impact on wheat susceptibility to cereal aphid pests. In addition, two summer morphs of cereal aphids, *Rhopalosiphum padi* and *Sitobion avenae*, held in culture at the James Hutton Institute were used. Furthermore, a control group of plants was grown without aphids (NA) along with the two treatment-controls (Ctrl1 and Ctrl2). The experiment followed a randomised block design with six replicate blocks each containing a

single replicate of the 36 treatment combinations (six bacterial treatments, three aphid treatments, two wheat varieties) with a total of 216 plants.

Seed preparation, germination and vernalization

Wheat seeds were placed into a beaker filled with distilled water and mixed slightly to disperse air. The seeds were left in water for 3 h and then surface sterilized by soaking in a solution of 2% (v/v) sodium hypochlorite for 15 min. Then seeds were rinsed several times using sterile distilled water to remove bleach.

Seeds were germinated in square Petri dishes (25 x 25 cm) sterilized by spraying with 70% ethanol that was allowed to evaporate. In each plate were placed two layers of filter paper dampened with sterile distilled water. Then seeds were placed on the filter papers with embryos facing downwards. Two layers of filter paper were placed on top of the seeds and dampened with sterile distilled water. The plates were closed and covered with aluminum foil before incubating at 15˚C for 3 days to promote germination (Karley *et al.,* 2011).

Plastic pots (50 mL) were sprayed with 70% ethanol and after ethanol evaporation they were filled with autoclaved artificial substrate containing 1:1:1 (by volume) washed sand:loam:grit. Germinated seeds were transferred into the substrate and vernalized at 6°C for 3 weeks.

After vernalisation, wheat plants were transferred into a rhizotube system developed at the James Hutton Institute that allows plant roots to be harvested intact to depths of 1 m (Karley *et al.,* 2011). Plants were grown using artificial soil prepared based on the chemical composition of the Sicilian soil where the bacteria were isolated. The main characteristics of the Sicilian soil were the high abundance of clay and sand, low concentration of organic matter and high pH and Calcium concentration. The artificial soil was prepared by mixing 40% washed sand, 40% vermiculite, 20% loam, 10 g/rhizotube of lime (calcium hydroxide) and 1 g/L of the slow release fertiliser Osmocote Exact Standard 3-4M (AICN, UK). The pH value of the prepared soil was 7.60. In order to reduce microbial contamination before inoculation with the bacterial treatments rhizotubes were first sprayed with 70% ethanol and air-dried before filling with substrate and the substrate was autoclaved before adding the fertiliser and being transferred into the rhizotubes (Figure 23).

Figure 23. Structure of the first experiment. *Wheat plants grown into rhizotubes in glasshouse conditions and connected to the watering system.*

Inoculum preparation and application

Bacterial treatments were prepared by transferring single colonies from each strain into 10 ml of Oxoid nutrient broth (Oxoid Ltd., UK). The cultures were incubated at 27°C for 24 h and centrifuged at 1118 x g for 10 min. The broth was discarded and the pellet was suspended in 10 ml of 10 mM $MgSO_4$. Each suspension was quantified by registering the optical density at 600 nm (OD_{600}) using the UV-1600PC spectrophotometer (VWR, UK). Based on the OD values a volume was transferred from each suspension to a glass bottle filled with sterile distilled water to reach a final concentration of 1x 10^6 colony forming units (CFU)/ml and a final volume of 500 ml. Aliquots of each suspension were mixed to give a concentration of 10^6 CFU/ml in 500 ml and used to prepare the autoclaved bacterial mixture.

Bacterial treatments were applied to the plants by adding 20 ml of the prepared bacterial suspension to each rhizotube using a 10 ml pipette with sterile tips. The plants were watered the day before and further watering was delayed until 48 h after the treatment in order to give the bacteria the time to colonize the roots.

Aphid cultures and application

Aphid nymphs belonging to the two species under study, *R. padi* and *S. avenae*, were reared in ventilated cups on one-week-old barley seedlings (*H. vulgare* cv. Concerto; growth stage

1.1–1.2 on the Zadoks scale). Cups comprised two Perspex cups (5 cm width × 15 cm depth) placed one inside the other with a 5 mm circular hole in the base of the inner cup. Barley seedling roots were placed into a 10 mm depth of water in the base of the outer cup, with the stem inserted through the hole in the inner cup. The cup surface was sealed with a mesh-ventilated lid. Aphid cultures were maintained at 18 ± 2 °C and 16 h:8 h (day:night) for 14 days, until adult stage was reached. Wingless adults were transferred, one per plant, onto the most recently expanded leaf on the main stem and contained using mesh-covered clip-cages (25 mm internal diameter) to prevent escape and allow monitoring of aphid growth and reproduction. As soon as the adults started reproducing, they were removed from the clip-cages using a fine paint brush and three nymphs (Gen1) were left on each plant inside the clip-cage (Figure 24).

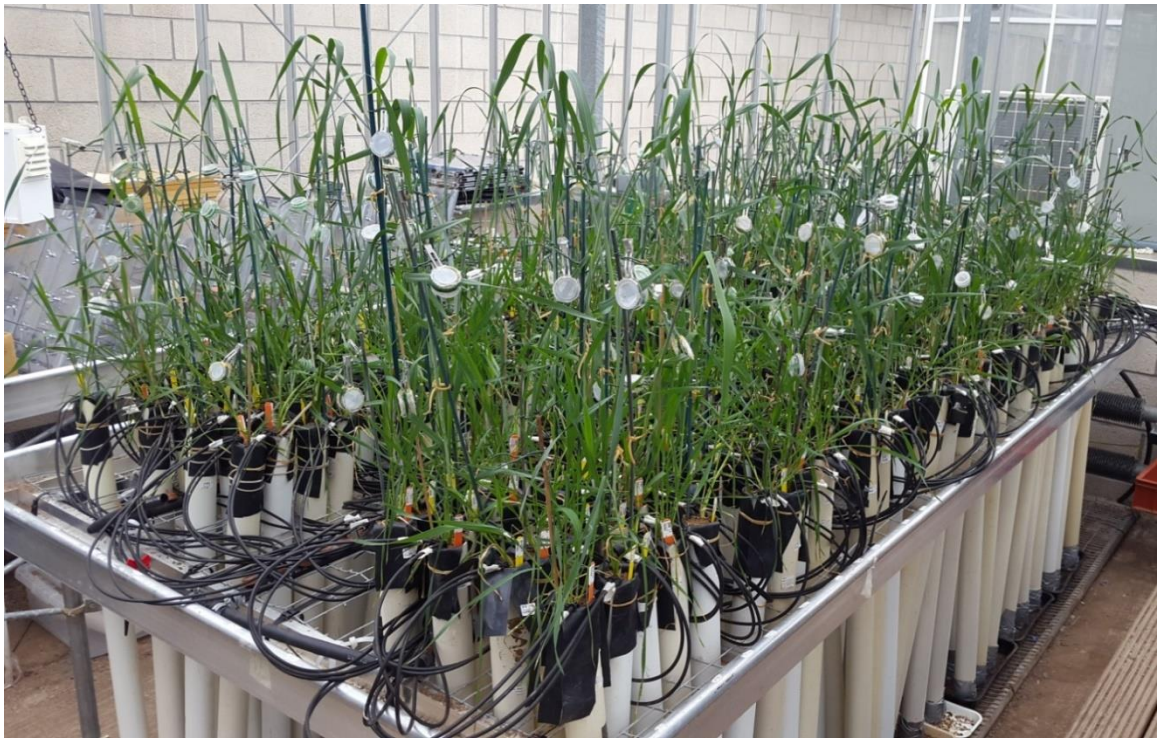

Figure 24. Structure of the second experiment. *The figure shows the plants grown in glasshouse during the second experiment involving aphids placed inside the clip-cages on the top leaf of wheat plants.*

Plant growth

Plant were measured before application of bacterial treatments at the beginning of tillering phase, at growth stage (GS) GS20 for Perciasacchi and GS23 for Cordiale (Zadoks *et al.*, 1974), in order to have a "time 0" as reference threshold to compare plant development after bacterial application. Plant measures at this stage included plant height of the main stem, number of tillers per plant and total number of leaves. Bacterial effect on plant growth was

evaluated by measuring plant development rate at three growth stages of the plant: end of tillering-stem elongation (time 1; GS23-25 and GS25-30 for Perciasacchi and Cordiale respectively), booting (time 2; GS47-49 and GS39-41 for Perciasacchi and Cordiale respectively) and flowering (time 3; GS65-69 and GS59-61 for Perciasacchi and Cordiale respectively). Measures of plant growth were taken in the same way as for time 0 followed by sampling in order to measure dry weight of aerial and root biomass. Number and length of ears were also measured during flowering phase. Biomass was measured at every time point by harvesting one block at time 1 and two blocks at time 2 and time 3. Plant biomass was measured using an analytical balance after collecting stems, leaves, ears and roots of each plant in paper bags that were dried at 70°C for 72 h.

Aphid performance

Treatment effects on aphid fitness were evaluated for the first generation (Gen1) nymphs produced on each plant by measuring their time/survival to adulthood, time to first reproduction and the number of nymphs (Gen2) produced after 12 and 14 days, for *R. padi* and *S. avenae* respectively. Plant biomass was also measured by collecting plant roots, stem, leaves and ears in paper bags that were dried at 70˚C for 72 h before measuring dry weight using an analytical balance.

Statistical analysis

Analysis of variance (ANOVA) was performed using R 3.5 (R Core Team, 2018)to evaluate treatment effects on plant performance, in terms of plant height and biomass production at different growth stages corresponding to times 0, 1, 2 and 3, and on aphid fitness. Briefly, the *aov()* function was used specifying treatment and cultivar as fixed effects and block/plant position as random factors in order to consider the effect of plant position. Effects of aphid morph (winged or apterous), on aphid fitness were also analyzed using a Linear Mixed-Effects model (LME) using the *lmer()* function and specifying treatment, wheat variety and aphid species as fixed effects and aphid morph, block/plant position and number of Gen1 aphids (between 1 and 3, varying with aphid survival) as random factors. Type II Wald X^2 from the package *car* and function *Anova* were used to evaluate the effect of treatments and pairwise contrasts using Tukey's multiple comparison procedure was calculated using package *emmeans*.

Results

Taxonomic identification of bacterial strains

BLAST alignment of the 16S rRNA sequences obtained from Sanger sequencing allowed the identification of the 24 potential PGPRs listed in Table XVII.

Table XVII. Sequenced bacteria isolated and screened for plant-growth promoting activities. *Results of Sanger sequencing; DSMZ/TRBA: human potential pathogens based in DSMZ database, level1= no risk; level>1= potential human pathogen. fw: forward primer coverage/identity; rv: reverse primer coverage/identity. ID: corresponding to the isolates listed in chapter 4, Table X.*

ID	Bacterial species	Coverage	Identity	DSMZ/ TRBA
Endophytes				
8	*Pseudomonas cerasi*	100%fw / 100%rv	98%fw / 98%rv	1
15	*Brevibacterium frigoritolerans*	100%fw / 100%rv	100%fw / 100%rv	1
20	*(Pseudo) Arthrobacter siccitolerans*	100%fw / 100%rv	98%fw / 99%rv	1
35	*Pseudomonas savastanoi*	100%fw / 99%rv	99%fw / 99%rv	1
39	*Pseudomona schlororaphis*	100%fw / 100%rv	99%fw / 99%rv	1
49	*Pseudomonas cedrina*	100%fw / 100%rv	100%fw / 99%rv	1
63	*Stenotrophomonas chelatiphaga*	100%fw / 100%rv	99%fw / 99%rv	1
84	*Acinetobacter calcoaceticus*	100%fw / 100%rv	100%fw / 100%rv	2
91	*Pseudomonas putida*	100%fw / 100%rv	99%fw / 99%rv	2
92	*Pseudomonas putida*	100%fw / 100%rv	98%fw / 99%rv	2
101	*Pseudomonas cedrina*	100%fw / 99%rv	100%fw / 100%rv	1
130	*Pseudomonas marginalis*	100%fw / 100%rv	100%fw / 99%rv	1
Root associated				
41	*Pseudomonas extremorientalis*	100%fw / 100%rv	99%fw / 98%rv	1
85	*Pseudomonas cedrina*	100%fw / 100%rv	99%fw / 99%rv	1
86	*Pseudomonas cedrina*	100%fw / 100%rv	99%fw / 99%rv	1
126	*Pseudomonas mucidolens*	100%fw / 99%rv	98%fw / 99% rv	1
138	*Pseudomonas helmanticensis*	100%fw / 100%rv	100%fw / 99% rv	1
159	*Pseudomonas japonica*	100%fw / 100%rv	99%fw / 99% rv	1
179	*Pseudomonas koreensis*	100%fw / 100%rv	100%fw / 99% rv	1
186	*Pseudomonas putida*	100%fw / 100%rv	100%fw / 99% rv	2

Bulk soil				
1	*Microbacterium maritypicum*	100%fw / 100%rv	100%fw / 100%rv	1
3	*Pseudomonas cedrina*	100%fw / 100%rv	100%fw / 98%rv	1
5	*Bacillus circulans*	100%fw / 100%rv	99%fw / 99%rv	2
7	*Pseudomonas vancouverensis*	99%fw / 100%rv	100%fw / 99% rv	1

Among them 13 strains were identified as potential human (score 2) or plant pathogens according to scientific literature and DSMZ/TRBA classification thus were excluded from further tests. The following 12 bacterial species were identified as non-pathogenic; therefore, they were used as plant treatments in glasshouse conditions: *Brevibacterium frigoritolerans*, *Pseudoarthrobacter siccitolerans*, *Pseudomonas chlororaphis*, *Stenotrophomonas chelatiphaga*, *Pseudomonas cedrina* (two strains), *Pseudomonas extremorientalis*, *Pseudomonas mucidolens*, *Pseudomonas helmanticensis*, *Pseudomonas koreensis*, *Microbacterium maritypicum* and *Pseudomonas vancouverensis*.

Bacterial activity on wheat growth

Analysis of variance on wheat growth performance indicated significant differences between the two wheat varieties under study in terms of plant height (ANOVA $F(1, 192)=510.348$, $p < 2.00E-16$) and plant biomass (ANOVA $F(1,83)=124.435$, $p=<2E-16$). Indeed, Perciasacchi plants were visibly taller than Cordiale plants even though the latter produced more tillers than the former variety.

The effect of treatment was not significant for plant height and root, leaf, ear or stem biomass (not shown), but there was a significant interaction between bacterial treatment and cultivar for ear biomass (ANOVA $F(13,83)=2.64$, $p=0.00392$) and stem biomass (ANOVA $F(13,83)=1.902$, $p=0.0414$) because the two wheat varieties showed contrasting responses to some bacterial inoculants. In particular, treatment T03 was associated with significantly smaller ear biomass (Tukey's HSD test, $p<0.05$) compared with Ctrl 1 (autoclaved inoculum) in Cordiale plants; a similar trend was observed for stem biomass (data not shown). Conversely, Perciasacchi plants showed a trend towards increased stem and ear biomass in response to the same treatment. In addition, trends in plant biomass in response to treatment T05 were similar to T03. By contrast, the response of the two varieties to T08 in terms of ear biomass showed the opposite trend, with higher values for Cordiale and lower values for

Perciasacchi compared with Ctrl1. No significant difference was shown between bacterial treatments and Ctrl2 (sterile water) (Figure 25).

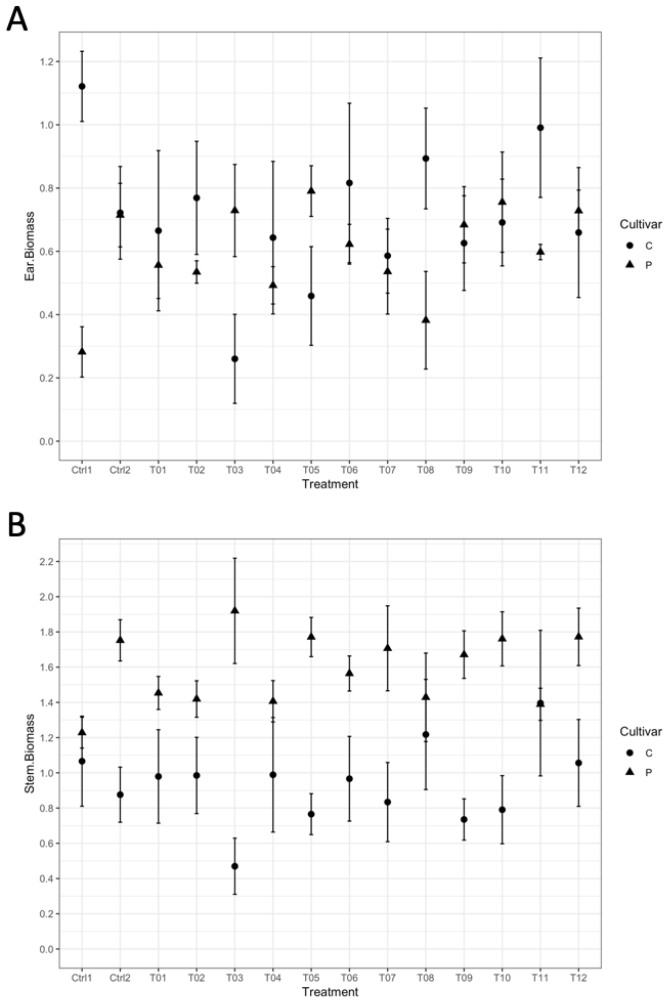

Figure 25. Ear biomass and stem biomass. *Mean values of ear (A) and stem (B) dry mass for Cultivar C (circle): Cordiale; Cultivar P (triangle): Perciasacchi; Values are means of n=8 plants and bars represent standard error of the mean.*

Interestingly, the two wheat varieties showed opposite reactions to the presence of the autoclaved bacterial components. Indeed, mean ear biomass values of Perciasacchi and Cordiale biomass were lowest (0.282 g) and highest (1.122 g) respectively in response to Ctrl1 (autoclaved inoculum) while ear biomass values associated with Ctrl2 (sterile water) treatment (0.715 g and 0.722 g in Perciasacchi and Cordiale, respectively) were similar for the two cultivars.

Bacterial effects on aphid fitness

Based on the results obtained in the first experiment, four treatments were chosen to test their effects on plant susceptibility to aphid pests. In more details, treatment T03 was chosen for its significant effects shown on wheat ear biomass. Treatments T05 and T07 showed similar trends to T03 on wheat stem biomass. In addition, *P. cedrina* and *P. extremorientalis* are reported in literature as beneficial species; therefore, these two treatments were further tested in the second experiment. Finally, T01 showed a neutral effect in all analysed data and it was added to the experiment as further control as a living inert bacterium. Aphid fitness did not vary significantly in response to the bacterial treatments T03, T05 and T07 in terms of aphid development time, production of winged morphs and reproduction rate. However, there were significant differences between the two wheat varieties: *R. padi* reproductive rate was higher on Perciasacchi compared with Cordiale ($F(1,101.66)=8.06$, $p=0.005$) (Figure 26). Aphid infestation had no significant effect on plant biomass of either wheat variety (data not shown).

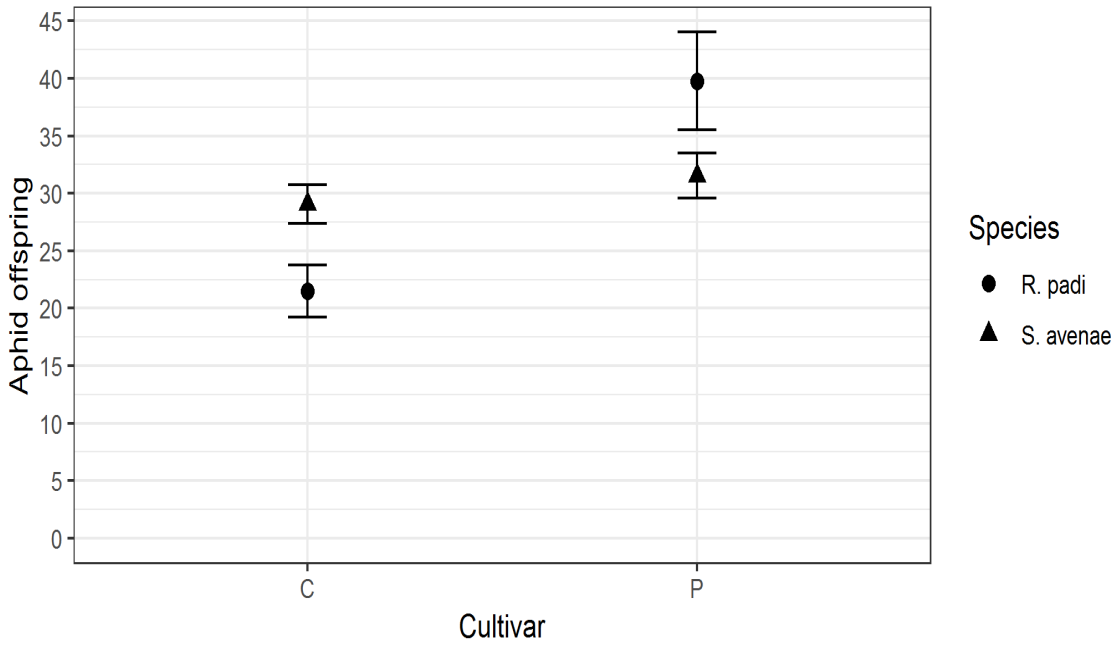

Figure 26. Aphid reproduction rate on the two wheat varieties. *The graph shows the No. of offspring produced by R. padi after 12 d (circle) and by S. avenae after 14 d (triangle) on Cordiale (C) and Perciasacchi (P) plants. Values are means of n=36 replicates and bars represent standard error of the mean.*

Discussion

This study has evaluated the effects of potential PGPR strains on plant growth and tolerance to aphid infestation on a modern and ancient wheat variety. Among the tested bacteria, *P. chlororaphis, P. cedrina* and *P. extremorientalis*were found to significantly affect wheat growth in terms of stem and ear biomass production. Consequently, these bacteria were further tested in a second experiment in order to evaluate their effect on aphid fitness.

Interestingly, the autoclaved bacterial mixture influenced plant growth producing contrasting responses from the two wheat varieties. This result suggests that bacterial components (peptides, lipopolysaccharides and cell wall components) or autoclaved culture metabolites (ACMs) might induce a defensive response in the plant even after thermic sterilization (Song *et al.,* 2017; Freeman, 2008) and indirectly affect plant growth. Therefore, differential responses shown by Perciasacchi and Cordiale to the autoclaved bacterial mixture and to the live treatment T03 (*Pseudomonas chlororaphis*) could be due to differences in resource management adopted by the two varieties. Indeed, the first level of plant defence mechanisms rely on the recognition of conserved molecular structures (elicitors) essential for microbial fitness and known as microbe associated molecular patterns (MAMP), such as cell wall components including proteins, carbohydrates and lipids (Newman *et al.*, 2013). However, defence mechanisms are expensive in terms of energy with an impact on plant growth and development. This might explain the significant decrease in ear biomass shown by Perciasacchi in presence of Ctrl1 and of Cordiale inoculated with T03 that could be due to the activation of defence mechanisms induced by dead and living bacterial components respectively. Interestingly, Cordiale seems to be stimulated by the presence of dead bacterial components as it showed a higher ear biomass compared with the sterile water control treatment (Ctrl2) (Figure 4.3).

On the other hand, Perciasacchi showed a stronger positive response to the presence of the live treatment T03 (*P. chlororaphis*) compared to Cordiale. Considering that T03 was originally isolated from internal tissues of Perciasacchi plants this result might not be surprising. However, the PGPR role of *P. chlororaphis* has previously been reported showing an increase in shoot and length as well as in the number of ears in wheat (Carlier *et al.,* 2008). Furthermore, some strains have been reported to act as biocontrol agents and as PGPR in drought conditions in previous studies (Egamberdieva, 2009; Johnsson *et al.,* 1998) therefore it might be interesting to carry out more detailed study of T03 to evaluate its potential

antagonistic effect against important wheat pathogens and the evaluation of its effects in drought stress conditions on wheat plants.

Finally, the higher fecundity of *R. padi* aphids on Perciasacchi plants compared to Cordiale suggests that plant genotype plays a role in aphid reproduction. This result is coherent with previous evidences of differential responses of aphid fitness depending on host genotypes in barley (Rowntree *et al.*, 2010) and wheat regardless of plant domestication level (Migui and Lamb, 2003). Plant genotype is also one of the main factors involved in recruiting plant-associated microbial communities which might explain the differential responses of the two wheat varieties inoculated with Ctrl1 and T03. Therefore, further experiments should be performed on other wheat varieties in order to evaluate the range of effectiveness of combinations of the tested bacteria on biotic stressors such as aphid infestation and on abiotic stress tolerance, such as drought.

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Chapter 6
Conclusions and future perspectives

The present work allowed to investigate the composition and activities of the microbial communities associated to ancient wheat landraces, Perciasacchi and Tumminia, in order to shed some light on their effects on plant growth and health. A multidisciplinary approach was used to study the complex network of composition and interactions involving the microbial communities and their host plant from different points of views. Hyperspectral reflectance data, acquired through proximal sensing approach, were first used to identify hyperspectral vegetation indices (HVIs) connected with wheat growth by correlation studies using plant height as growth indicator. The results obtained allowed to select four HVIs strongly correlated with wheat growth, including well characterized indices such as Vi_{opt}, PSSRcar and PSSRa and a newly calculated HVI, Slope, related to leaf maturation. Afterwards, these data showed interesting connections with the results obtained from the study of the endophytic fungal communities and bacteria beneficial activities associated to the ancient wheat landraces.

Indeed, molecular analyses, using high throughput sequencing, showed the effects of geographic location and agricultural practices as well as wheat genotype on the composition of the wheat-associated fungal endophytic communities. Furthermore, the interactions observed among the detected genera suggested the presence of microbial-mediated resistance of wheat varieties. In this framework, the selected wheat growth-related HVIs showed interesting direct correlations with some fungal genera, including *Alternaria*, *Protomyces* and *Bullera*, indicating that these endophytic genera might include beneficial species for wheat development.

Moreover, culture-dependent methods allowed to identify a group of bacterial isolates performing beneficial activities for plant growth. Some of these activities, in particular NH_4^+ production, were correlated with the hyperspectral vegetation indices Slope, Vi_{opt}, PSSRa and PSSRcar. Considering the association of Vi_{opt} with the leaf nitrogen content, PSSRa and PSSRcar with the leaf pigments content and the mutual correlation between these indices, the results obtained are coherent with each other.

During glasshouse experiments, one of the tested bacteria isolated from Perciasacchi, *Pseudomonas chlororaphis*, showed significant responses, in terms of head biomass, from the two tested wheat varieties, Perciasacchi and Cordiale. In particular, Perciasacchi showed positive responses to the presence of this bacterial treatment, whereas Cordiale, the modern UK variety, showed a negative response. This observation confirms the importance of the host genotype when plant-microbe interactions are involved. Therefore, it might be useful to further test the bacterial strains selected in this work on other wheat varieties in order to evaluate the responses of a wider range of hosts. Interestingly, the autoclaved bacterial mixture produced significant effects on plant growth as well. This observation is encouraging for the possibility of using bacterial-derived active molecules as an advantageous alternative to the application of living bacteria for improving plant performance and the safety of field operators. In addition, despite the bacterial isolates tested did not show any advantage in terms of plant tolerance against the cereal aphids *Rhopalosiphum padi* and *Sitobion avenae*. even in this case host genotype showed an important role on plant interaction against aphid pests.

The multidisciplinary approach used in this work produced encouraging results for investigating the relationships between plant-associated microbial communities, their activities and their roles towards their host. In particular, the correlations observed between the HVIs, fungal endophytes and bacterial activities are encouraging and lay the foundations for using hyperspectral reflectance data to identify beneficial plant-associated microorganisms directly in the field. In addition, the detection of HVIs, here performed in small scale using proximal sensing, could be transferred to the remote sensing technology with the acquisition of hyperspectral data from wider field surfaces using drones, aircrafts or satellites.

This approach might offer interesting applications in the field of precision agriculture for several purposes such as the selection and propagation of highly performing plants associated with beneficial microorganisms or a targeted sampling of plants for the isolation of beneficial strains for field application or even for identifying the plants of interest for ecological studies.

Nevertheless, further studies are still needed to investigate a wider range of wavelengths and wheat varieties, in order to confirm and strengthen the correlation resulted between HSVs and plant physiological conditions. In addition, it has been demonstrated that complex microbial mixtures produce stronger effects on the plant in terms of disease resistance and growth promotion, leading the focus of future studies to the concept of “synthetic communities” rather than application of single inoculant (see Finkel et al., 2017 for review). Therefore, it

would be interesting to perform further experiments in glasshouse conditions using combinations of the PGPRs selected in this work.

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