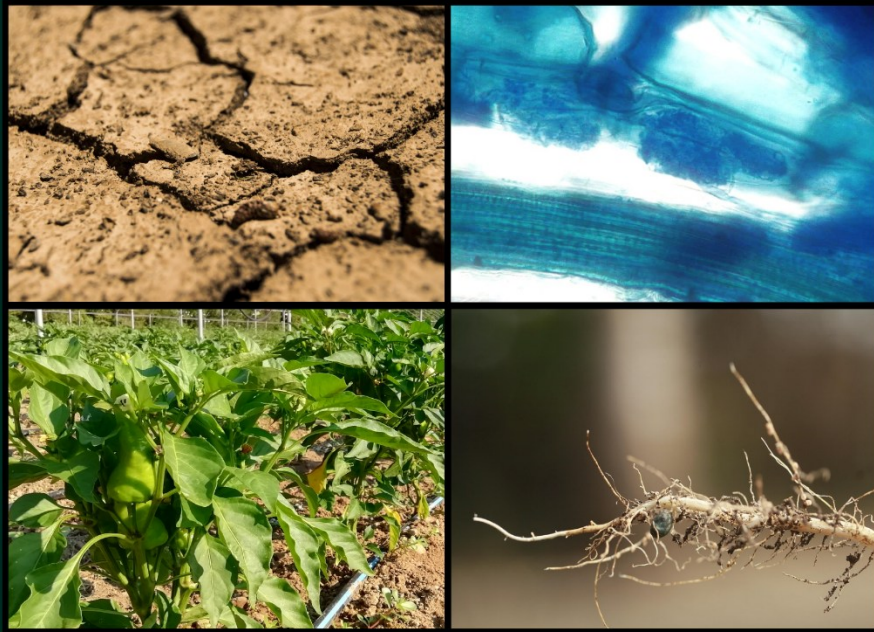




University of Sassari
Department of Agricultural Science
Doctoral School in Agricultural Science

PhD program in Desertification and Land Degradation
Cycle XXXVIII

**Exploring the potential of microbial communities and bio-based solutions
in improving plant tolerance and resilience to stresses**

Alice Calvo

Academic tutor: **Prof. Quirico Migheli**

Co-Tutor: **Dott. Raffaella Maria Balestrini**





University of Sassari

Department of Agricultural Science

Doctoral School in Agricultural Science

PhD program in Desertification and Land Degradation

Cycle XXXVIII



Exploring the potential of microbial communities and bio-based solutions in improving plant tolerance and resilience to stresses

Alice Calvo

Academic tutor: **Prof. Quirico Migheli**

Co-Tutor: **Dr. Raffaella Maria Balestrini**

Summary

Introduction	1
Climate change and its effects on the agricultural sector	1
Definition of desertification	1
Drought as the main consequence of climate change scenario	3
Soil properties and their effects on plant water availability	4
Drought impacts on plants growth and physiology	6
Best management practices related to drought management	8
Bio-based solutions as management practices to improve plant growth	10
Arbuscular mycorrhizal fungi	12
Plant growth-promoting bacteria	16
Mycorrhizosphere	18
The importance of soil health	20
Volatile organic compounds as soil health indicators	23
Metabarcoding analysis as a tool to study soil and root associated microbiota	26
Databases for taxonomic classification	29
Tools for microbial taxonomic assignment	30
Aim of the Thesis	34
Chapter I	37
Interactions between an arbuscular mycorrhizal inoculum and the root-associated microbiome in shaping the response of <i>Capsicum annuum</i> “Locale di Senise” to different irrigation levels	38
Abstract	39
Keywords	39
Introduction	40
Materials and methods	41
Experimental design	41

Sampling procedure	41
Soil moisture and extracellular enzyme activities	41
Ecophysiological measurements and agronomic parameters	42
Assessment of mycorrhizal colonization in roots	42
Biochemical markers and phytohormones quantification in leaves	42
Antioxidant and nutritional analysis on pepper fruits	43
Metabarcoding analysis	43
Statistical analysis	44
Results	44
Soil chemical-physical characteristics	44
Soil enzyme activities	44
Ecophysiological measurements, plant development and production	45
AM fungal colonization in pepper roots	45
Analysis on biochemical markers and phytohormones in leaves	46
Analysis of antioxidant compounds and micronutrients in fruits	48
Fungal and bacterial communities in bulk soil and roots over time	48
Discussion	53
The impact of the irrigation treatments on pepper ecophysiological and agronomic parameters	53
The role of the AM fungal inoculum on plant responses to irrigation treatments	53
The impact of the irrigation and the AM fungal inoculum on the soil and root-associated microbial communities	54
Acknowledgements	56
Author Contribution	56
Funding	56
Data availability	56
Declarations	56
Open Access	56
References	56
Chapter II	61

Impact of bacterial inoculations on *Pisum sativum* L. biometric parameters and associated bacterial and AM fungal communities under semi-arid field conditions in Tunisia

Abstract	62
Introduction	62
Materials and methods	63
Experimental design	63
Bacterial inoculation	64
Biometric parameters and nitrate content in pea leaves	64
Soil sampling	64
Statistical analysis	64
Metabarcoding	64
Results	67
Biometric parameters	67
Soil chemical-physical properties	67
Soil AM fungal community	67
Soil bacterial community	68
Comparison between the two years and correlation between AM fungal and bacterial taxa	70
Discussion	70
Bacterial inocula positively affected plant biometric traits in both pea varieties	70
Soil AM fungal and bacterial alpha diversity increased in presence of the bacterial inoculation	72
Different putative PGPB were associated with specific AM fungal groups	73
Conclusions	74
CRedit authorship contribution statement	74
Funding	74
Declaration of competing interest	74
Acknowledgments	74
Data availability	74
References	74
Chapter III	77

Legume choice matters: different effects on *Brassica napus* agronomic performance and root-associated bacterial communities in intercropping

systems	78
Abstract	78
Keywords	79
Introduction	79
Materials and methods	80
Experimental design	80
Soil sampling for chemical-physical analysis	81
Measurement of agronomic parameters in <i>B. napus</i>	81
DNA extraction, sequencing and metabarcoding analysis on roots and rhizospheric soil samples	81
Statistical analysis	82
Results	82
Soil physical–chemical analyses and climatic data	82
Agronomic parameters	82
Analysis of bacterial communities	82
Discussion	89
The two intercropped legume species differentially affect <i>B. napus</i> agronomic parameters especially in the dry growing season	89
Rhizosphere and root microbial communities were influenced by the adopted legume species in the intercropping systems	89
Conclusions	91
Acknowledgements	91
Funding	91
Data Availability	91
Declarations	91
Competing interest	91
References	91
Chapter IV	96

Microbial diversity and cover plants in de-sealed urban soil as strategies for mitigating anthropogenic volatile organic compounds _____ 97

Abstract _____ 98

Keywords _____ 98

Introduction _____ 99

Materials and methods _____ 100

Site and soil sample collection _____ 100

Experimental design and sampling _____ 100

Assessment of arbuscular mycorrhizal (AM) fungal colonization _____ 100

Assessment of soil microbial biodiversity through metabarcoding _____ 100

Volatile organic compound (VOC) analysis _____ 102

Statistical analysis _____ 102

Results _____ 103

Soil physical-chemical parameters at T1 _____ 103

Arbuscular mycorrhizal (AM) fungal colonization _____ 103

Composition and structure of soil microbial communities _____ 103

Soil VOC signature _____ 107

Correlation between VOC emissions and microbial communities _____ 109

Discussion _____ 109

Application of AM fungal inoculum might enhance microbial diversity and soil health status _____ 110

The interaction between *C. cardunculus* and AMF contributed to AVOC biodegradation and restoration of soil health status _____ 110

Synergistic effects within the mycorrhizosphere of AMF-inoculated plants contributed to soil nutrient availability _____ 112

Conclusions _____ 112

Acknowledgement _____ 112

Declarations _____ 112

Author contributions _____	112
Data availability _____	112
Funding _____	112
Declarations _____	112
Conclusions of this Thesis _____	118
The use of bio-based solutions in arid and semi-arid environments _____	118
The importance of specific microorganism-microorganism interaction in improving plant growth _____	118
The role of legumes as a natural source of nitrogen in sustainable agriculture _____	119
Innovative approaches in soil microbial composition evaluation and monitoring _____	120
Final conclusions _____	120
References _____	122
Sitography _____	136
Appendix A _____	137
Correlation between microbial communities and volatile organic compounds in an urban soil provides clues on soil quality towards sustainability of city flowerbeds _____	138
Abstract _____	138
Introduction _____	138
Materials and methods _____	139
Soil sampling _____	139
VOC analysis _____	140
Dynamic headspace sampling of volatile compounds _____	140
Thermal desorption gas chromatography–mass spectrometry (TD-GC–MS) analysis of volatile compounds _____	140
Metabarcoding analysis _____	140
DNA extraction and sequencing _____	140
Bioinformatic and statistical analysis _____	141

Results	141
Soil physical-chemical features	141
VOC analysis	141
Microbial community assessment	143
Correlation between VOC emissions and microbial communities	145
Discussion	146
Soil from flowerbed harbored higher microbial diversity compared to the urban park	146
VOCs in soil from flowerbed originated from anthropic and biological origin	146
Microbial communities and VOC emissions from soil seem to be associated with the close tree species	149
Conclusions	150
Data availability statement	150
CRediT authorship contribution statement	150
Declaration of competing interest	150
Acknowledgments	150
Appendix A. Supplementary data	150
References	151

Impact of two *Erwinia* sp. on the response of diverse *Pisum sativum* genotypes under salt stress

Abstract	154
Keywords	154
Introduction	155
Materials and Methods	156
Isolation of the bacterial strains	156
Estimation of PGP activities in vitro	156
Biocontrol activity of bacterial strains against phytopathogens	157
Molecular characterization of bacterial strains	157
Seed sterilization and plant growth conditions	157
Bacterial inoculation in pea plants	157
Salt application	158
Biometrical and physiological measurements of plants	158

Biochemical analysis of <i>P. sativum</i> stress markers	158
Quantitative gene expression analysis in <i>P. sativum</i> leaves	158
Statistical analysis	159
Results	160
Characterization of bacterial strains	161
<i>P. sativum</i> biometric and physiological parameter evaluation	161
Biochemical analysis of <i>P. sativum</i> stress markers	161
RT-qPCR results	161
Gene expression and biochemical data correlation	163
Discussion	165
Characterization of bacterial strains	165
Effects of salt stress on biometric and physiological parameters of <i>P. sativum</i> plants	167
Stress marker evaluation	167
Gene expression and correlation analysis	168
Conclusion	169
Supplementary Information	169
Acknowledgements	169
Author contributions	169
Funding	169
Data availability	169
Declarations	169
Conflict of interest	169
Open Access	169
References	169
Ringraziamenti	173

Introduction

Climate change and its effects on the agricultural sector

In 2022, the Intergovernmental Panel on Climate Change (IPCC) reported a world-wide global temperature increase of 1.1 °C due to climate change scenario (Ray Biswas and Rahman, 2023). Climate change is defined as a long-term variation in one or more climate variables, such as temperature and rainfall patterns (Burroughs, 2007) and it directly impacts agricultural yield and food security (Nelson et al., 2009). Rising temperatures cause crop yield decreases due, on the one hand, to reduced water availability and, on the other hand, to increased water requirement, derived both from higher plants evapotranspiration rates and higher soil water evaporation (Lakhia et al., 2024). Consequently, food prices increase and this, combined with yield reduction, has a negative effect on agricultural production (Nelson et al., 2009). Such situation leads to food insecurity, in which food availability, access, utilization and stability over time are compromised (Mirzabaev et al., 2023).

In this context, measures able to mitigate climate change are required. Mitigation strategies mainly rely on the reduction of greenhouse gas (GHG) emissions due to the human activities (Ray Biswas and Rahman, 2023). Besides these measures, boosting agricultural productivity under climate change scenario also depends on human climate change adaptation, defined as a set of anthropic actions able to adapt to the new climatic conditions and to improve ecosystems resilience while reducing their susceptibility (Owen, 2020).

Definition of desertification

One of the major threats under climate change scenario is desertification (Intergovernmental Panel On Climate Change, 2022). The United Nations Convention to Combat Desertification (UNCCD) in 1994 defined the term desertification as “*land degradation in arid, semi-arid and dry sub-humid areas resulting from various factors, including climatic variations and human activities*”. Specifically, “land degradation” is used to indicate the loss of biological or economic productivity and complexity of agricultural areas resulting from land exploitation (UNCCD, 1994). Desertification results from the interaction among different environmental factors (*e.g.*, drought, soil erosion, and vegetation-cover loss) and affects agricultural fields, urban areas, and

natural resources. Its effects include soil depletion, vegetation loss, and land degradation (Huang et al., 2020; UNCCD, 1994).

Climate change exacerbates this phenomenon by altering rainfall patterns, temperatures, and wind regimes (Huang et al., 2020). The Mediterranean Desertification Land Use Model (MEDALUS) identified four key factors driving land degradation: climate, soil, land management, and vegetation (Kosmas et al., 1999). However, this model was developed exclusively for the Mediterranean region and does not consider social, economic, or political dimensions (Huang et al., 2020). New models should be based on the integration of environmental and anthropogenic aspects, understanding the differences of ecosystem services available in the affected regions and countries and assessing desertification phenomenon at different scales (Verstraete et al., 2009). Desertification should be in fact monitored locally, but upscaled progressively to global observations, allowing data collection and monitoring at each level. Different aspects should be taken into account by considering the level of observation, allowing a projection of the desertification phenomenon at the specific observed scenario *e.g.*, local, regional, national or global (Verstraete et al., 2009).

Climate change is reflected in a higher frequency of extreme events such as droughts, heatwaves, floods, and storms (Muheki et al., 2024). One of the main hotspots of climate change scenario is North-East Africa (Choi et al., 2023; Kimutai et al., 2025; Muheki et al., 2024; Taye and Dyer, 2024). This area is particularly subjected to climate change, because it suffers from water scarcity, highly depends on rainfed agriculture and lacks on resources (Choi et al., 2023). From 2020 to 2023 this area suffered from the worst drought event in 40 years related to the absence of rainfall events (Kimutai et al., 2025). Due to the presence of several countries characterized by different climatic conditions, the effects of climate change highly depend on the affected region, showing different patterns and magnitude levels (Taye and Dyer, 2024). As a result, the environmental and the human systems are currently compromised, showing an increased vulnerability and lowest adaptive capacity to climate change (Kimutai et al., 2025). This implies the need to translate large-scale predictive models at country level to strengthen the adaptive capacity of each country (Taye and Dyer, 2024), which represents one of the main issues in facing climate change scenario in drought-prone environments.

Drought as the main consequence of climate change scenario

Drought represents the primary consequence of climate change (Wanders et al., 2015). It is defined as a slow-onset environmental hazard characterized by prolonged periods of low rainfall (months or years), and it slowly evolves into a complex phenomenon. It can be classified in meteorological, hydrological and agricultural drought. Meteorological drought occurs when a reduction in rainfall events and rainfall total amount are observed in a short or long period of time (Quiring, 2009). This can be defined as the simplest form of drought, since it depends only on rainfall trends. A complex phenomenon is the so-called pseudo-drought or physiological drought in which the water is available, but the plant is not able to fully access the reservoir due to external factors such as salinity, low soil temperature and floodings that decrease the overall plant water

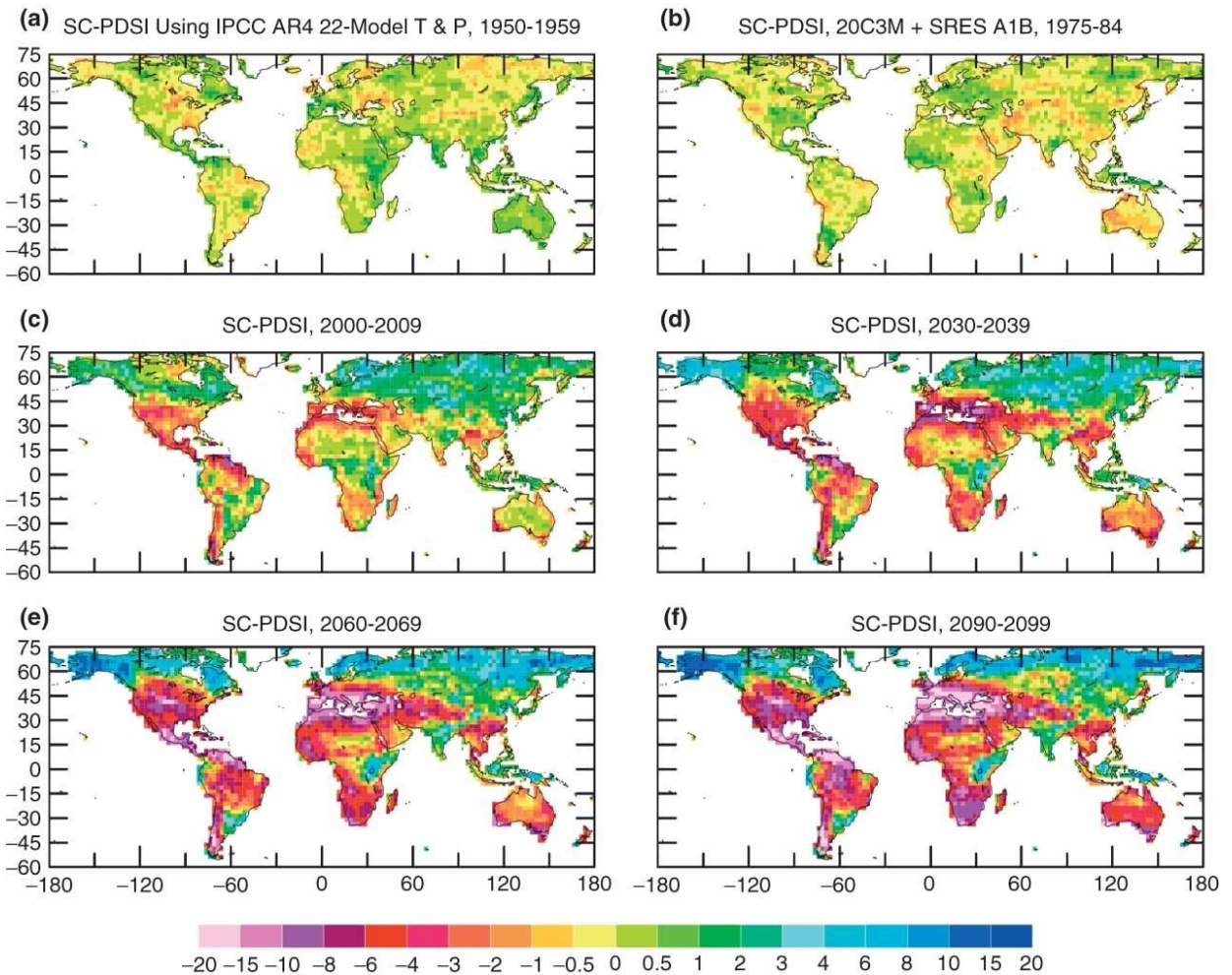


Figure 1: Drought global spread for years (a) 1950–1959, (b) 1975–1984, (c) 2000–2009, (d) 2030–2039, (e) 2060–2069, and (f) 2090–2099. Red to pink areas are extremely dry (severe drought) conditions while blue colors indicate wet areas relative to the 1950–1970 (Dai et al., 2011).

uptake capacity (Salehi-Lisar and Bakhshayeshan-Agdam, 2020). On the other hand, the absence of rain may compromise the hydrological system, increasing the so-called hydrological drought (Hisdal et al., 2024). In fact, hydrological drought is defined as a state of water shortage in the hydrological system, that includes rivers, lakes, groundwater and reservoir water depletion (Hisdal et al., 2024). Drought is currently a widespread phenomenon that will increase in the next years (Gu et al., 2020). **Figure 1** shows a projection of global drought, highlighting its increase from 1950 and the worsening of this phenomenon in the next future (Dai, 2011). Precipitations and soil moisture are undergoing a decrease and one of the most affected areas is North Africa.

Soil properties and their effects on plant water availability

Soil composition plays a crucial role in water availability. Soil can be classified based on its texture, porosity, structure and aggregation, bulk density, soil organic matter (SOM), salinity and hydraulic conductivity. Each property can affect the others in a multi-partite interaction (Hartmann et al., 2020). Soil texture describes the relative presence of sand, clay and loam in the soil and can be classified using the USDA texture triangle. Basically, soil is classified based on the different percentages of these three components (Soil Science Division Staff, 2017). In soils, porosity indicates the space occurring between soil particles, defined as pores. Pores can be further classified in micropores and macropores based on their size. Soil structure is defined by the association among soil particles. The primary particles ($< 20 \mu\text{m}$) aggregate forming the so-called microaggregates up to $250 \mu\text{m}$ of diameter. Microaggregates can further aggregate in macroaggregates (Rabot et al., 2018). Bulk density concept merges the concepts of soil structure and porosity. In fact, it is defined as “the mass of a unit volume of dry soil (105°C)”, including both pores and solids and thus indicating the actual total soil porosity (FAO, 2006). Soil organic matter indicates the content in organic carbon and represents the primary source of nutrients for plants (Lehmann and Kleber, 2015). Soil salinity measures salts concentration in soil and is physically represented by electrical conductivity (EC) (Shahid et al., 2018). Salts in soil usually derived from soil mineral salts and are classified in anions and cations based on their charge. The main anions that can be find are Cl^- , SO_4^{2-} , HCO_3^- , NO_3^- and CO_3^{2-} , while the main cations are represented by Na^+ , Ca^{2+} , Mg^{2+} and K^+ (Tanji, 2002). Hydraulic conductivity in soil is the result of the interaction among all soil properties and defines how water can be stored in the soil and how it can be translocated to the other environmental components (Hartmann et al., 2020). Water

movement in soil towards a specific direction depends on gradients in water potential, while hydraulic conductivity controls the amount of water that can move along these gradients (Bittelli, 2010; Koehler et al., 2023). Soil water potential represents the energy status of water in the soil and is determined by the combined effects of matric, gravitational, osmotic, and pressure components. As soil becomes drier, soil water potential decreases, reflecting stronger water-retention activity performed by soil particles (Affortit et al., 2024). At the same time, also hydraulic conductivity declines, limiting the capacity of the soil to supply water to the other environmental components, such as plant roots. Plant water uptake therefore depends on both soil properties (Carminati and Javaux, 2020). On the other hand, plant water potential reflects the energy status of water within plant tissues and decreases as the water is translocated from the soil through the roots, the xylem, the leaves and atmosphere (Carminati and Javaux, 2020). Water uptake occurs when plant water potential is lower than soil water potential (Affortit et al., 2024). However, as soil dries and hydraulic conductivity declines, the soil system is not able to translocate the water required to sustain the flux needed by the plant to meet transpiration demand, leading to a rapid decrease in plant water potential (Koehler et al., 2023).

Soil properties influence plant water uptake through different mechanisms occurring at soil-root level. Variations in pore size, presence and connectivity, depending on native soil structure and external phenomena such as soil compaction, can decrease plant water uptake due to the decrease in hydraulic conductivity (Goldberg-Yehuda et al., 2024; Peralta Ogorek et al., 2025). High bulk density can limit root elongation and thus the exploration of soil volumes, thereby reducing the total absorptive surface available for water uptake (Peralta Ogorek et al., 2025). Low levels in SOM affect not only plant nutrient uptake but also soil water adsorption. In fact, high levels of SOM can positively affect properties such as soil pores, structure and soil water-holding capacity, sustaining water uptake (Fageria, 2012). Thus, SOM plays a crucial role in enhancing plant resistance to drought. Salinity effect on water uptake is mainly represented by osmotic stress, as high concentrations of salt lower the osmotic component of soil water potential independently of the actual soil moisture content, reducing the gradient driving water movement from soil to roots, even when water is physically available (Lu and Fricke, 2023). In combination, these processes modulate the spatial and temporal availability of water at root-soil level, affecting plant water uptake and inducing plant-responses to drought.

Drought impacts on plants growth and physiology

The main plant disorders caused by drought can be classified in reduction of plant growth and productivity, water balance disorders, altered minerals and nutrients uptake and imbalance in lights absorption and carbon fixation (Liu et al., 2023). In plants, drought ultimately leads to osmotic stress (Xiao and Zhou, 2023). The osmotic stress directly impacts on cell turgor (Xiao and Zhou, 2023; Zhu, 2002) and it is caused by cell water-imbalances that can negatively affect cell activities (Chen and Jiang, 2010). Under osmotic stress, the water contained in the cell, especially in the vacuole, is transported outside to balance the internal/external ion concentration required for the correct cell function. This leads to cell water-deficit, with consequent loss of turgor and cell death (Zelm et al., 2020).

One of the major consequences of drought is photosynthesis reduction, which leads to chlorophyll degradation and to Reactive Oxygen Species (ROS) accumulation (Balasubramaniam et al., 2023). Other effects may be represented by alterations in stomatal conductance, stomatal cell damage, as well as chloroplast deformations, but also by alterations in the synthesis and function of photosynthesis-related proteins (Zahra et al., 2022). In fact, ROS produced under these conditions may degrade or inactivate cells proteins, especially the ones involved in photosynthesis, but also the ones involved in signaling, membrane stability and metabolic processes (Choudhary et al., 2020). As observed for proteins, lipids may be damaged by ROS activity. Osmotic stress can induce changes in lipid composition, as well as the proportion of different types of lipids in the cell membrane and their role in the signaling pathways (Guo et al., 2019). Under drought stress, plants regulate several response mechanisms, including ROS signaling, phytohormones production and osmolytes synthesis (Ahluwalia et al., 2021; Bashir et al., 2021; Sato et al., 2024). Plants are able to detoxify ROS via a ROS-scavenging activity, producing antioxidant enzymes as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione peroxidase (GPx) as well as antioxidants such glutathione and ascorbic acid (Sato et al., 2024). Among phytohormones, abscisic acid (ABA) and ethylene play key roles: ABA induces stomatal closure, reducing CO₂ uptake and photosynthesis, while ethylene limits transpiration (Ahluwalia et al., 2021; Sato et al., 2024). Moreover, drought triggers also the production of osmolytes able to reduce water loss from cells as proline, trehalose, and glycine betaine, sugars and polyamines (Ahluwalia et al., 2021).

Plant phenotype may be affected by drought. In fact, plants reduce shoot biomass to reduce their metabolic demand and to reduce transpiration, while increasing root formation to enhance soil water uptake (Bashir et al., 2021). Interestingly, plant drought-scavenging mechanisms often involve the same molecules that can cause plant growth reduction and senescence during drought. ROS signaling is commonly adopted by plants to signal different types of stress, including drought. At the same time, ROS accumulation can cause decreased rate of photosynthesis, lipid peroxidation, protein mutations, DNA alterations and cell death (Huang et al., 2019). In the same way, stomatal closure prevents water loss from the plants but may also cause drop in CO₂ content in photosynthetic cells, leading to photosynthesis reduction and to alterations in leaf morphology and anatomy under prolonged drought, with consequent reduction of the photosynthetic efficiency (Branch et al., 2024; Flexas and Medrano, 2002).

As previously stated, plants are able to adapt their phenotype to different stresses. During drought, the root-shoot ratio increases, shoot biomass is reduced to limit metabolic demand, and the loss of water via transpiration. Root emission increases to better allow soil exploration and water resources exploitation but at the same time drought also causes root thinning. This process may lead to root cells damage and alteration in the root system architecture (Sun et al., 2024; Tenorio Berrío et al., 2022; Zhou et al., 2018). Absciscic acid (ABA) and ethylene are two common messengers in drought-stress that also regulate plant evapotranspiration. Increases in ABA and ethylene content inhibit plant growth, seed germination, and guide the process of plant senescence, leading to cell death (Chen et al., 2021). The production of osmolytes allows cell turgor maintenance, ROS detoxification and integrity of the membranes. However, this process is often ineffective during rapid drought phenomena and is highly genotype-dependent (Ahluwalia et al., 2021).

In summary, drought triggers several physiological, biochemical, and morphological responses in plants. The adaptive mechanisms, such as ROS scavenging, hormonal regulation, osmolyte accumulation and phenotypic adaptation, mitigate drought impact on plant but often rely on the same processes that drive stress progression. For this reason, plant response to drought is a very balanced system, in which each mechanism needs to be highly regulated.

Best management practices related to drought management

Among the different mitigation and adaptation strategies that can be adopted, a major role is played by climate-smart agricultural (CSA) practices (Lipper et al., 2014). These practices primarily aim to enhance agricultural sustainability, reduce greenhouse gas emissions, and increase farmer-based systems adaptation to climate change (Autio et al., 2021; Teklu et al., 2022). Climate-smart agricultural practices are especially promising for smallholder farmers; however, their adoption rate remains low (Kangogo et al., 2021). One reason, as suggested by Giller et al. (2011), is the time required before some practices show measurable yield benefits. For example, no tillage shows positive results in terms of agricultural production only after several years and, considering the high level of poverty, farmers are often reluctant to sacrifice higher short-term yields in favor of long-term benefits (Giller et al., 2011). Additionally, farmers often rent agricultural land from different landowners each year, and the rented land is usually small, not allowing the stop the agricultural production for soil recovery without compromising the total production (Cotula et al., 2004; IFAD, 2016). Crops rotation with legumes is another example of key CSA practice, that not only provides food but also nourishes the soil. However, its adoption is hindered by understocked markets. Additionally, the implementation of CSA faces challenges due to the low production of crop residues necessary for mulching, leading to reduced crop production and biomass availability (Giller et al., 2011). It is important to note that most CSA practices, including no tillage, use of cover crops, and crop rotation, fall under the category of Conservation Agriculture (CA), which aims to minimize soil disturbance, to maintain permanent soil cover with residues or cover crops, and to promote appropriate crop rotation (Diop et al., 2022). Among the different CSA practices, an important role in facing climate change scenario is represented by intercropping (Okoronkwo et al., 2024). Intercropping is an agricultural practice in which multiple crop species are simultaneously grown in a single field (Dowling et al., 2021). It is largely adopted in climate change prone-scenarios due to its economic and simple application (Burgess et al., 2022). Intercropping optimizes space use, reduces competition for resources, suppresses weeds, enhances nutrient and water-use efficiency, and improves soil nutrient status (Huss et al., 2022). It can also limit pathogens via allelopathic compounds production and physical barriers (Huss et al., 2022). A specific method of intercropping is the push-pull technology, a practice that exploits the presence of a pest-repellent

(*Desmodium* sp.) and a pest-attractive forage plant in the cultivated field. The pest-repellent crop is intercropped with the desired productive crop *e.g.*, cereals, while the pest-attractive one is cultivated on the field border. This scheme provides the main crop defense from pests that are naturally attracted on the field borders (Adesina et al., 2023). Intercropping often relies on the use of leguminous crops that can increase N reservoirs in the soil, reducing the dependence on chemical fertilizers and the consequent negative impact of their application on the environment (Zampieri et al., 2025). Moreover, legume-based intercropping has been proved to enhance soil microbial diversity, positively improving plant growth (Zampieri et al., 2025). For all these reasons, this practice represents an easy, economic and sustainable approach to adapt crops production towards climate change scenario.

An example of CSA practice adoption in drought-prone environments - such as Africa - is represented by the project Climate Smart WATER Management and Sustainable DEVelopment for Food and Agriculture in East Africa (WATDEV, <https://www.watdev.eu/>), which aims to improve the adaptation of CSA practices, defined as best management practices (BMPs), in East Africa. The project targets four Countries known to be subjected to climate change and desertification phenomenon, namely Egypt, Ethiopia, Kenya and Sudan (Aboukota et al., 2024; Choi and Eltahir, 2023; Lawrence et al., 2023; Sinore and Wang, 2024). The objective of the project is to find and adapt a set of BMPs in each Country able to improve water and land management under the climate change scenario. These BMPs have been selected from the existing scientific knowledge and proposed to the main actors of the project, a group of stakeholders (SHs) including farmers, academics and politics. The presence of a multi-layered background, determined by the different knowledge and field of interest of each SH, has been fundamental to define the needs and gaps in terms of putatively adoptable BMP. Each scientifically pre-documented BMP has been evaluated through a questionnaire aimed to define its potential of adoption in each Country. The scientific knowledge has been further implemented based on each SH response *via* ad-hoc questionnaires with a final selection of a set of BMPs able to mediate the current scientific knowledge and SHs perspectives for each Country. After this process, these BMPs have been implemented in local case-study areas, in order to evaluate the suitability in upscaling their adoption at national level.

Bio-based solutions as management practices to improve plant growth

Traditional agricultural practices are currently threatening environmental health. The use of chemical fertilizers, pesticides and the intensification of agriculture are causing serious damages to the soil compartment (Sandrini et al., 2022). Soil microorganisms are directly impacted by these environmental constraints and are subjected to a negative selective pressure that leads to their decline (Sandrini et al., 2022). These conditions are worsened by the current climate change scenario, which increases the overall negative impact on plant growth.

Plants naturally share the soil environment with different microorganisms able to improve plant growth and health (Berendsen et al. 2012). This complex interaction may be exploited in improving plant fitness under abiotic stress conditions. Beneficial microbes inhabiting the rhizosphere and root tissues can activate stress-protective pathways that mitigate the adverse effects of environmental stresses, often through several synergistic mechanisms. The chemical environment of the rhizosphere plays a major role in modulating symbiotic associations (Balestrini et al., 2024). Root-released metabolites can serve as nutrients for specific microbes, as antimicrobial compounds to suppress potential pathogens, and as chemical signals to attract targeted microbial partners. Moreover, plants can actively regulate the relative abundance of specific exudate compounds, thereby influencing the composition and structure of rhizosphere microbial communities (Balestrini et al., 2024).

In the context of current agricultural production, new methods to improve sustainability without impacting crop production are needed. The adoption of bio-based solutions relying on beneficial microorganisms is emerging as a promising tool that can be classified as a group of BMPs (Agrimonti et al., 2021). Beneficial microorganisms are a wide group of microorganism that have a positive impact on plant growth and that can be used to improve agricultural production (Balestrini et al., 2024). Bio-based solutions stem from biological sources of different origins, including microbial sources (Priya et al., 2023). Microbial-based solutions can exploit natural microorganisms selected from a specific environment *e.g.*, soil, root or rhizosphere-inhabiting microorganisms, or synthetic microbial communities derived from *in vitro* cultured strains and then employed *in vivo* (Leggieri et al., 2021). Several studies have proved microbial-based solutions as effective in improving plant growth (Arcidiacono et al., 2025; Laranjeira et al., 2021; Li et al., 2024). However, the efficiency of this strategy largely depends on several

parameters. In fact, the native soil microorganisms may positively or negatively interact with the inoculum, increasing or lowering its efficiency in boosting plant fitness (Li C. et al., 2024). Moreover, also soil chemical-physical composition has a direct impact on inoculum efficiency (Juyal et al., 2021; Pettersson and Bååth, 2013). For example, soil structure, pH and nutrient availability are fundamental parameters in shaping soil microbial composition (Custódio et al., 2022). An additional important point is that positive plant-microorganism interaction depends on both plant and microorganism species. In fact, plants positively interact only with specific microbial species (van der Putten et al., 2013).

Considering these data, understanding the interactions among plant-microorganisms in soil is fundamental in improving bio-based solutions efficiency. Field experiments are needed to fully evaluate all the environmental variables that may interact with both native and synthetic microbial communities (Fadiji et al., 2024). Moreover, field experiments mimicking natural conditions, help the scientific community to better understand the progressive impact of climate change on agriculture (Maaz et al., 2025). Lastly, the use bio-based solutions in field experiments is needed to understand how the natural microbiota interacts with the used consortia and how to adapt the inoculum formulation to the specific field conditions (Fadiji et al., 2024; Liu et al., 2022).

Arbuscular mycorrhizal fungi

Beneficial microorganisms are mainly divided into two groups, including fungi and bacteria (Kumawat et al., 2022). Among fungi, a group of beneficial soil fungi is represented by arbuscular mycorrhizal fungi (AMF), a monophyletic group of biotrophic obligate, multinucleate, and non-cultivable organisms (Bonfante and Genre, 2008) that form symbiotic relationships with ~ approximately 80% of terrestrial plants (Lanfranco et al., 2016).

The taxonomy of AMF has always been and continues to be a matter of debate and has changed several times over the years. The first study that taxonomically classified AMF dates back to 2001, when for the first time AMF were placed in a separate phylum called Glomeromycota, distinguishing them from Zygomycota (Schüßler et al., 2001). After this first classification, AMF taxonomy remained an object of controversy. In 2016 and 2017, two studies changed AMF classification, placing AMF in the phylum Mucoromycota and in a new sub-phylum called Glomeromycotina (Spatafora et al., 2017, 2016). Currently, AMF classification is again matter of debate as a re-classification in the phylum Glomeromycota has been proposed by Rosling et al. (2024). This study affirms that AMF are phylogenetically very close to Mucoromycota but possess unique features at genome level that justify their classification in a separate phylum.

Plant and AMF are able to interact *via* symbiotic associations. The process of symbiosis between AMF and plants, in which the fungus enters plant roots cells, is based on a series of coordinated steps (**Fig. 2**). Prior to direct contact between the fungal hyphae and the root cell, diffusible signaling molecules, produced by both organisms, mediate the mutual recognition. Arbuscular mycorrhizal fungi detect potential host plants by sensing strigolactones released from roots. These molecules trigger a series of pre-symbiotic responses, including fungal spores germination, elongation and branching of hyphae, enhancement of mitochondrial activity in the developing mycelium, activation of effector gene expression and synthesis of plant-directed signaling compounds known as Myc-factors (Balestrini et al., 2024). Myc-factors consist mainly of chitin-derived structures which are recognized as microbe-associated molecular patterns (MAMPs). When plant receptors recognize Myc-factors, they initiate the common SYMBiosis (SYM) signaling pathway, which activates symbiosis-related genes and remodels cellular architecture, ultimately leading to AMF-plant contact (Balestrini et al., 2024). When the fungal

hypha reaches root cells, it forms hyphopodia, an AMF specialized attachment structure that allows the contact between the fungus and plant root cell (Lanfranco et al., 2016). Then, penetration in the root cortex occurs *via* intercellular or intracellular mechanisms. After colonizing the cortex, AM fungal hyphae enters single plant cell and develops into complex, highly branched structures called arbuscules. Arbuscules are surrounded by plant plasma membrane, that together with the arbuscule membrane, form the interface for nutrient exchange (Boyno and Demir, 2022; Genre et al., 2024; Perotto and Balestrini, 2024). The plant membrane

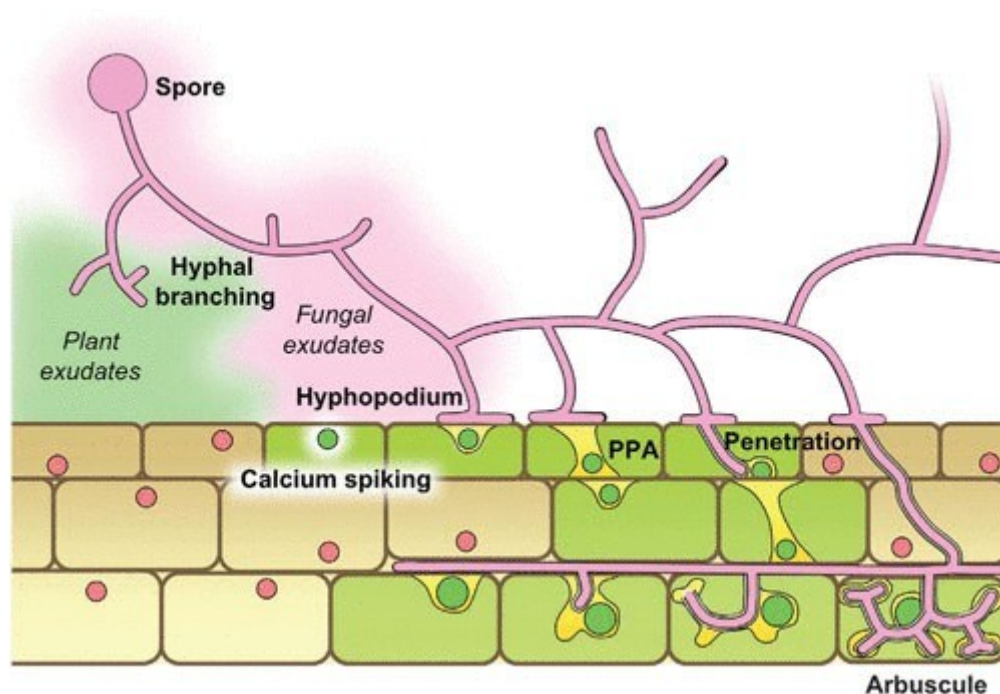


Figure 2: Root colonization by AMF and relative AM fungal structures (Bonfante and Genre., 2010).

remains intact forming an invagination around the fungal hyphae, separating them from the cytoplasm of the plant (Perotto and Balestrini, 2024). This process is similar to the pathogenic process in which pathogenic fungi, such as powdery mildew agents, smut fungi and some oomycetes, exploit plant resources *via* an invagination of the plant plasma membrane in which a specialized hypha called haustoria mediates the transfer of nutrients and virulence factors without damaging both organisms membranes (Kwaaitaal et al., 2017).

Arbuscular mycorrhizal fungi can improve plant water and nutrient uptake via their hyphal system (Smith et al., 2000; Smith and Read, 2010) and the activation of diverse plant molecular pathways (Berruti et al., 2016; Rouphael et al., 2015). Among the different nutrients provided by the fungus to the plant, the most relevant is represented by phosphorus (P) (Chen et al., 2018). Phosphorus is an essential element that is often unavailable for plants, due to its deficiency in soils and to the presence of phosphate forms that cannot be adsorbed by plants (Vance et al., 2003). Plants adsorb P mainly as orthophosphate and soil reservoirs are mostly composed of P assimilated into organic compounds or precipitated with cations (Chiu and Paszkowski, 2019). Arbuscular mycorrhizal fungi are able to extend their hyphal system beyond the P depletion zone in which plant roots develop, accessing new reservoirs and translocating P into the plant thanks to the presence of P transporters, in exchange of sugars and fatty acids (Chiu and Paszkowski, 2019).

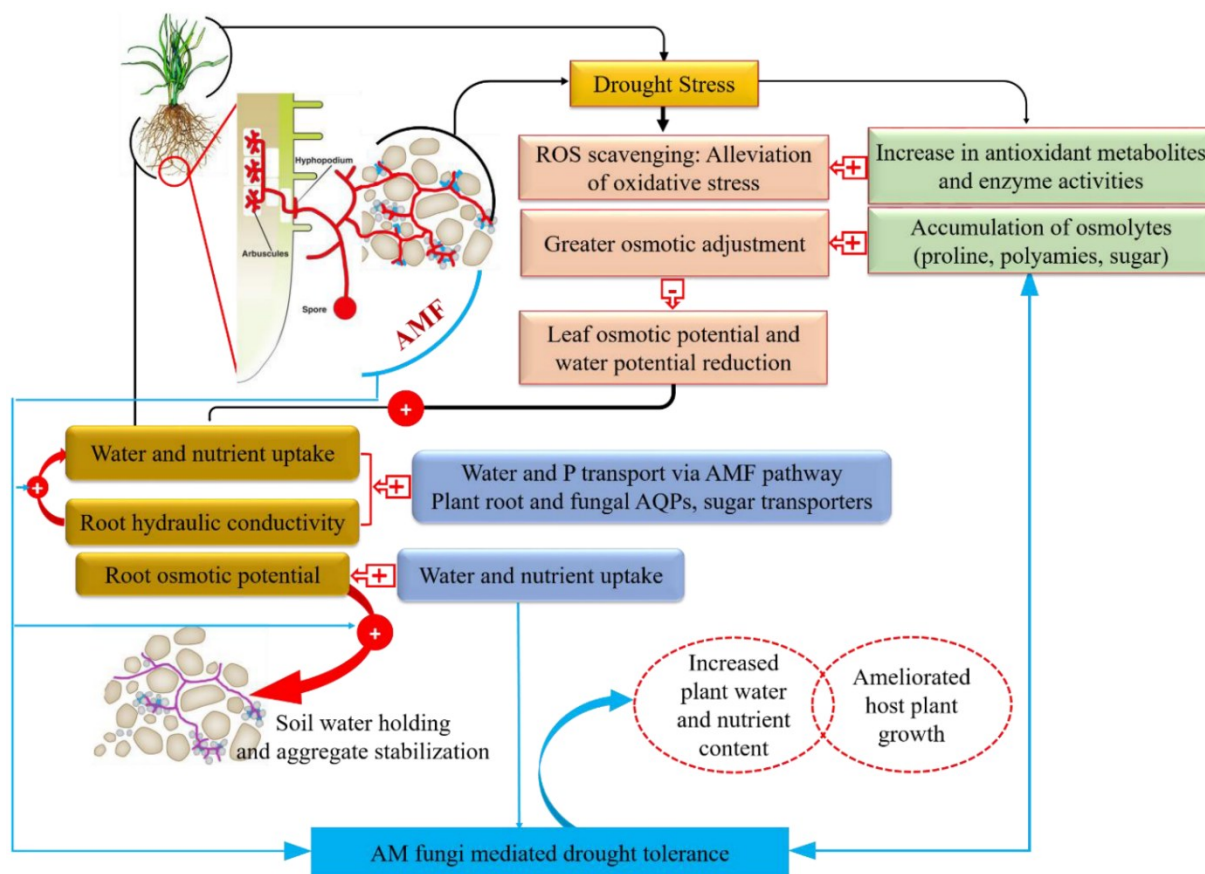


Figure 3: Schematic representation of the different mechanisms at the basis of the AMF-mediated improvement in plant tolerance to drought (Bahadur et al. 2019).

Besides the role in nutrient and water uptake, AMF can improve plant resistance to different stresses, such as drought (**Fig. 3**). Several studies have documented AMF's positive role in improving plant growth under stress conditions, including drought and salinity (Augé, 2004, 2001; Augé et al., 2015), as well as in improving resistance against plant pathogens (Azcón-Aguilar et al., 2002; Gianinazzi and Schüepp, 1994; Weng et al., 2022). As already mentioned, AMF can improve water and nutrient uptake *via* their hyphal system, thus allowing the plant to explore a larger volume of soil and water (Chitarra et al., 2016; Kakouridis et al., 2022). Arbuscular mycorrhizal fungi directly enhance root apparatus, improving root length, lateral root emission and increasing root surface area (Nie et al., 2024). They are also able to improve soil structure stability, alleviate ROS toxic effects on the plant, regulate phytohormone production, and regulate stress-related gene expression in plants (Wang X. et al., 2024). Among the different phytohormones that can be triggered in plants by AMF, indole-3-acetic acid (IAA) promotes plant root elongation, while high levels of abscisic acid (ABA) enhance plant response to drought (Wang X. et al., 2024). An example of stress-related genes upregulation is represented by aquaporins and water channel genes, structures that allow a facilitate plant water uptake (Nie et al., 2024). To maintain the correct plant cell osmolarity under osmotic stress, AMF also enhance osmolyte production, such as glucose, fructose and sucrose and proline (Nie et al., 2024). It has been documented that AMF can be used in bioremediation, thanks to their ability to sequester pollutants and heavy metals from the soil, immobilizing toxic compounds in lipid bodies inside fungal cells, or reduce them to less toxic forms (Lenoir et al., 2016). This highlights the valuable role of AMF not only in enhancing plant resilience to stresses, but also in supporting environmental restoration and contributing to human health.

Plant growth-promoting bacteria

Bacteria that have a positive effect on plants are known as plant growth-promoting bacteria (PGPB) (Bashan and Holguin, 1998) and include a wide range of phyla, as Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Gammaproteobacteria and Firmicutes (Ahmad et al., 2008). Beneficial bacteria inhabiting the soil improve plant growth via several different mechanisms, such as soil micropores enhancement due to the secretion of exopolysaccharides (Philippot et al., 2024). Moreover, a positive effect on soil nutrient uptake has been documented due to bacteria ability in converting inaccessible nutrient forms in accessible ones or in producing molecules able to mitigate water stress effect (Ali et al., 2022; Caddell et al., 2020). Plant growth-promoting bacteria enhance plant growth producing hormones, solubilizing phosphates, fixing nitrogen, increasing the water and minerals uptake, enhancing root development, increasing the plant enzymatic activity and suppressing pathogens (de Andrade et al., 2023). Additionally, PGPB may

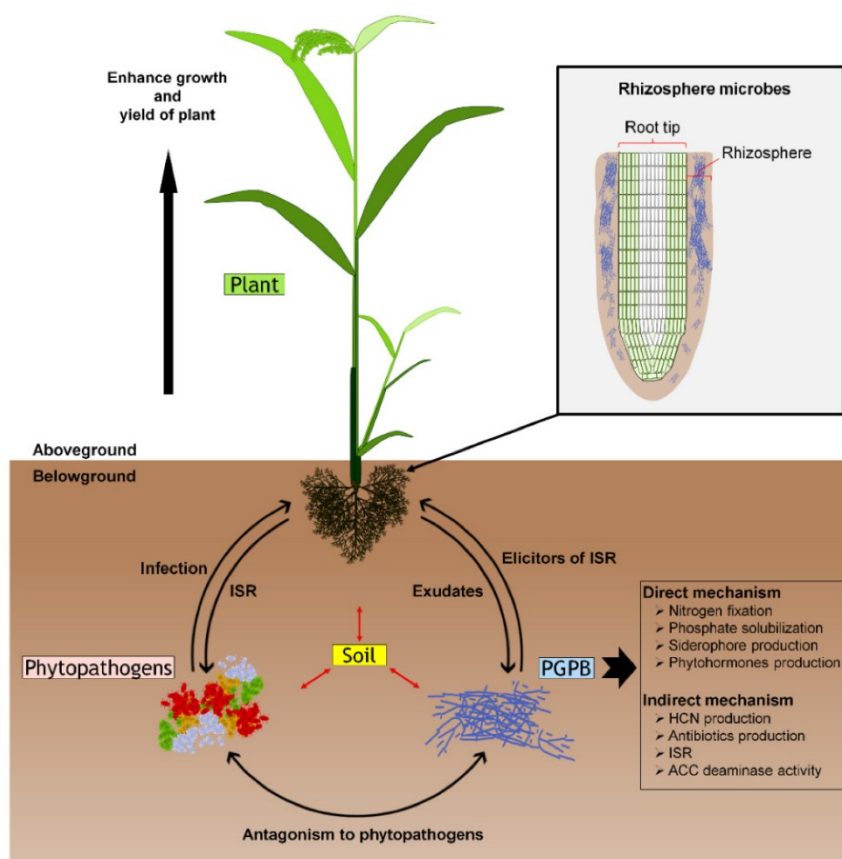


Figure 4: Plant growth-promoting activities performed by PGPB (Ngalimat et al., 2021).

“help” other beneficial microorganisms, indirectly improving plant performance (de Andrade et al., 2023; **Fig. 4**).

The most common PGPB are represented by species belonging to *Azospirillum*, *Bacillus*, *Azotobacter*, *Pseudomonas*, *Serratia*, *Enterobacter*, *Rhizobium* and *Streptomyces* genera. *Azospirillum* has been reported to improve iron nutrition in plants due to its ability to synthesize siderophores (Delaporte-Quintana et al., 2020). Among PGPB able to fix atmospheric N, the most important group is represented by *Rhizobium* sp., which represents the primary source of N in legume land-based systems (Zahran, 1999). Additionally, also *Azotobacter* sp. have been reported as N- fixers, thereby increasing the N reservoir for plants (Sumbul et al., 2020). *Bacillus* species are well known for their role as PGPB, enhancing plant growth *via* several mechanisms, such as hormones production, nutrient solubilization and ACC deaminase production. Additionally, they produce antibiotics, induce systemic resistance and siderophores (Kelbessa et al., 2023). *Pseudomonas* sp. is another well-known genus encompassing several PGPB, such as strains of *P. fluorescens* (David et al., 2018), which enhance plant growth through hormone production, siderophore synthesis, and acting biocontrol activities that boost plant resistance to pathogens (David et al., 2018). Several *Serratia* species are recognized as biocontrol agents, producing a wide range of antibiotic molecules (Kshetri et al., 2019). Some *Enterobacter* species are able to produce hormones, ACC deaminase, siderophores and antimicrobial compounds, as well as to fix atmospheric N, thus promoting plant growth in several ways (Jha et al., 2011). *Streptomyces* genus includes members able to improve nutrient release in soils, such as P, Fe and Mg. Furthermore, several members produce hormones and antibiotic molecules thanks to a highly differentiated secondary metabolism (Sousa and Olivares, 2016). Overall, PGPB may improve plant resistance to different types of stress, positively influencing plant fitness leading to better adaptability to stress conditions. Plant growth-promoting bacteria also directly enhances plant defense mechanisms, for example, *via* the production of ROS and antioxidant molecules (Chieb and Gachomo, 2023).

Mycorrhizosphere

The complex interaction among plants, AMF and bacteria occurs at different spatial levels. While there is a direct relation between AMF and plant roots, bacteria may interact directly with both the host plant and AMF, resulting in an indirect promotion of plant fitness (Sangwan and Prasanna, 2022). Bacteria, able to improve N fixation, P solubilization, producing antibiotics and hormones related to plant growth, as IAA, are defined as PGPB while bacteria able to directly improve AMF growth are defined as mycorrhizal helper bacteria (MHB) (Giovannini et al., 2020, **Fig. 5**). Mycorrhizal helper bacteria are strictly associated with AMF spores or mycelium (Battini et al., 2017). The term mycorrhizal helper bacteria has been adopted for the first time by Frey-

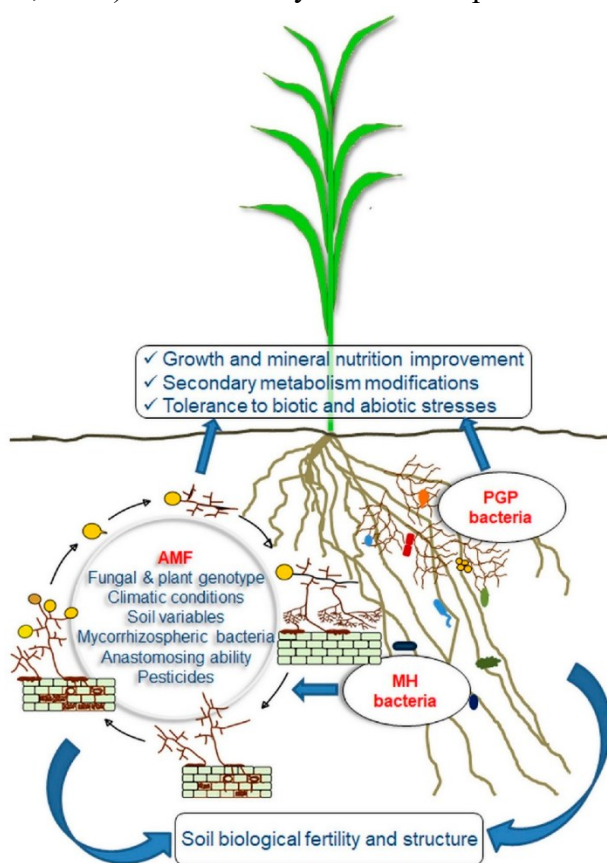


Figure 5: Interaction between AMF and MHB in enhancing plant growth (Giovannini et al., 2020).

Klett et al. in 2007 but the first who described this phenomenon was Jean Garbaye in 1994, with the isolation of bacterial communities directly from the AMF hyphae. Mycorrhizal helper bacteria may either have a direct contact with AM fungal hyphae or may occupy the mycelium surrounding zone (Garbaye, 1994). This space represents the interface between bacteria and AM fungal hyphae, in which the communication between these microbial groups occurs, similarly to what observed for plants in the rhizosphere. For this reason, this interface is called “hyphosphere” (Andrade et al., 1998; Faghihinia et al., 2023; Garbaye, 1994).

The bacteria involved in the tri-partite interaction with plants and AMF belong to several genera (Sangwan and Prasanna, 2022) such as *Lysobacter*, *Pseudomonas*, *Cellvibrio*, *Chondromyces*, *Flexibacter*, *Arthrobacter*, *Sinorhizobium*, *Streptomyces*, *Bacillus*, *Paenibacillus*, *Herbaspirillum*, *Massilia*, and *Rhizobium* (Turrini et al., 2018). These bacteria improve AMF

growth and, therefore, plant fitness, *via* different mechanisms, such as P solubilization (Sbrana et al., 2022), upregulation of P transporters (Jin et al., 2024), but also improving hyphal growth, arbuscule formation, sporulation, hormone regulation and copper sequestration (Sangwan and Prasanna, 2022). These intimate interactions are reported to have not only an effect on the plant but also on shaping the soil and rhizosphere microbial community (Trivedi et al., 2020). It has been proved that MHB suppresses plant fungal pathogens *via* competition mechanisms, improving plant nutrient status, shaping the rhizosphere microbiota, changing root morphology, inducing systemic resistance in plants, and shaping root exudates to reduce pathogen growth (Bharadwaj et al., 2012).

The importance of soil health

Human health is directly related to all environmental components' health,, such as plants, animals, and microorganisms, as well as the ecological niches that they occupy (Banerjee and van der Heijden, 2023). During the convention promoted by the Wildlife Conservation Society in 2004, the concept “One World, One Health” has been promoted and the Manhattan Principles, have been stipulated (Yamada et al., 2014, <https://www.oneworldonehealth.org/>). The goal of these principles was to address the whole ecosystem sustainability, preventing biological and ecological losses, considering all the multidisciplinary and multi-actors agents directly or indirectly able to affect the different environmental components as responsible of this action. *“Only by breaking down the barriers among agencies, individuals, specialties and sectors can we unleash the innovation and expertise needed to meet the many serious challenges to the health of people, domestic animals, and wildlife and to the integrity of ecosystems. Solving today’s threats and tomorrow’s problems cannot be accomplished with yesterday’s approaches”* (<https://www.oneworldonehealth.org/>).

In this scenario, soil represents a fundamental ecosystem shared by the different organisms. Additionally, soil interacts with all the other environmental systems, as atmosphere, biosphere,

lithosphere, and hydrosphere (Lehmann et al., 2020). Soil is defined as the natural system composed of minerals and organic material that provides a living environment for the organisms (Voroney, 2007). Given the complexity of this system, it might be defined as soil habitat, including both the non-living components and living ones. All the components of this system can affect each other through multiple interactions that occur over time and depend on water availability (Voroney, 2007, **Fig. 6**).

Soil management is fundamental to allow agricultural production: the anthropogenic impact on soil is reflected in soil degradation, erosion, loss of organic matter, of structure and water, the increase in salinity or in pollutant concentration, as well as in loss of biodiversity (Kibblewhite et al., 2007). Therefore, maintaining soil health is fundamental.

The US Department of Agriculture defines soil health as “*the continued capacity of soil to*

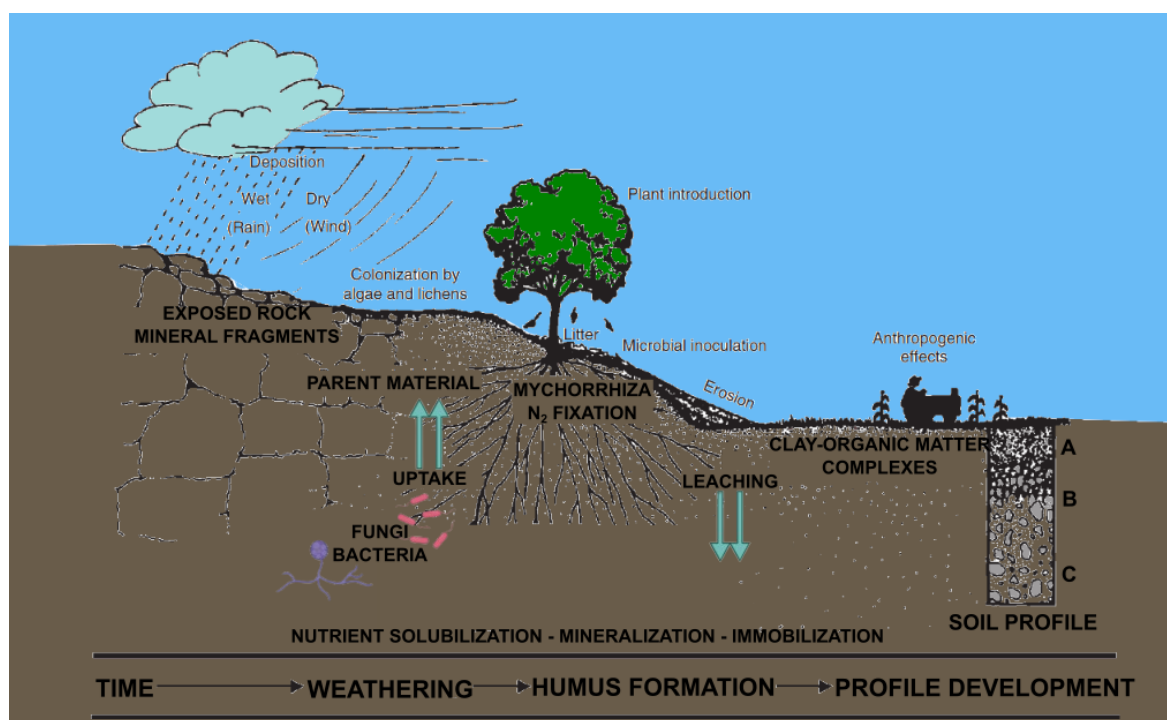


Figure 6: Soil habitat and its components (Voroney, 2007, modified).

function as a vital living ecosystem that sustains plants, animals, and humans” (<https://www.nrcs.usda.gov/conservation-basics/natural-resource-concerns/soils/soil-health>).

Soil health is a concept related to the interactions among the biological, chemical, and physical components of the ecosystem that influence soil functioning, whose objective is maintaining the

resilience and ecological integrity of the entire ecosystem (Lehmann et al., 2020). Soil health is not a static concept, but changes depending on natural and anthropogenic phenomena affecting all the environmental components. In agriculture, maintaining soil health is reflected in not prejudicing other ecosystem services (Kibblewhite et al., 2007). Nowadays, soil health should be monitored, especially considering the climate change scenario, which increases the impact on the previously described soil health indicators.

Several methods have been established to address soil health evaluation, through chemical, physical, and biological indicators (Cardoso et al., 2013; Usharani et al., 2019). Chemical indicators refer to soil chemical parameters as pH, soil organic matter, nutrient concentration and cation exchange capacity (CEC) (Cardoso et al., 2013). Physical indicators are related to soil's ability to optimize the associated hydrological processes. These indicators include texture, bulk density, stability and porosity of aggregates (Usharani et al., 2019). Biological indicators are referred as the organisms whose presence, absence, increase and decrease, as well as their biological function and behavior provide clues on the current state of the environment (Pulleman et al., 2012). These indicators are usually evaluated through a score-based system (Nunes et al., 2021). One example of these methods is the Soil Management Assessment Framework (SMAF). This index provides a score from 0 to 1, integrating different indicators, ranging from biological to chemical and physical ones (Nunes et al., 2021). The major problem with score-based systems is that they are primarily built on small datasets that collect soil data from limited regions. Therefore, they cannot be easily applied at global scale (Nunes et al., 2021). Recently, different global models have been created to investigate soil health at global scale as the SEMWISE (Deel et al., 2024) and the SoilHealthDB (Jian et al., 2020). These tools consider a higher number of soil health indicators collected in several countries, but they are difficult to use for non-trained users.

These data highlight the need for a multi-level approach, enabling the use of high-sensitive, complex methods on a global scale, as well as more feasible models that, although less precise, can be easily used by non-specialists, allowing the user to establish a proxy of soil health at the local level.

Volatile organic compounds as soil health indicators

A new sustainable approach to monitor soil health involves screening for volatile organic compounds (VOCs) (Kuchikata et al., 2024). Volatile organic compounds are defined as compounds containing carbon, excluding carbon oxides, that can volatilize (Mangotra and Singh, 2024). These compounds belong to two distinctive groups, including AVOCs, of anthropogenic derivation, and BVOCs, produced by biological activities (Sillo et al., 2024). Most of the human activities may contribute to AVOC accumulation and these compounds are naturally present in several products, leading to soil contamination (Mangotra and Singh, 2024). Anthropogenic VOCs include mostly alkanes, alkenes, aromatic compounds, oxygen- and nitrogen-containing compounds (Reimann and Lewis, 2007).

Biogenic VOCs can be produced by plants and microorganisms, affecting both soil and atmosphere composition (Tang et al., 2019). In particular, soil represents both a source and a sink of BVOCs, due to the presence of biological activities related to organic residues decomposition and microbial metabolisms, therefore acting as reservoir and as nutrients source for both plants and microorganisms (Abis et al., 2020; Owen et al., 2007). Among BVOCs, isoprene, monoterpenes and methanol are the major chemical compounds produced by plants (Loreto et al., 2014). At root level, the production of BVOCs may mediate several processes such as the interaction with AMF, boosting plant resistance to abiotic stresses (Asensio et al., 2012).

Microorganisms inhabiting urban soils are also able to produce BVOCs, including several classes of molecules such as alcohols, ketones and terpenes (Sillo et al., 2024, 2022). Both bacteria – such as *Pseudomonas* spp., *Bacillus* spp., *Staphylococcus* spp., *Serratia* spp., *Stenotrophomonas* spp. – and fungi – such as *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp., *Trichoderma* spp., *Tuber* spp., - produce a wide range of BVOCs (Insam and Seewald, 2010). Main VOCs produced by bacteria belong to esters, ketones, terpenes, alkenes, alcohols, benzenoids, pyrazines, and acids (Yang et al., 2024). Fungi produce BVOCs from both primary and secondary metabolism in a combination of aldehydes, hydrocarbons, heterocycles, ketones, alcohols, thioalcohols, thioesters, phenols, benzene derivatives and cyclohexanes (Morath et al., 2012). Each microorganism produces a distinct BVOCs pattern. The presence of specific BVOCs in soil and their screening allows the identification of soil microbial communities (Kanchiswamy

et al., 2015). The role of BVOCs in nature is diversified (**Table 1**). Microorganisms may use these compounds to communicate (Guo et al., 2021), to inhibit pathogens or competitors (Oufensou et al., 2025; Rani et al., 2023), to modulate aggregation and biofilm formation (Schmidt et al., 2016) and to change the environmental conditions to promote their own growth or to allow nutrient availability in soils (Masteling et al., 2022). These compounds can also positively affect plant growth (**Fig. 7**), acting as biofertilizers (Chandrasekaran et al., 2022), inducing plant systemic resistance (Montejano-Ramírez et al., 2024) and improving soil health (Baloch et al., 2024).

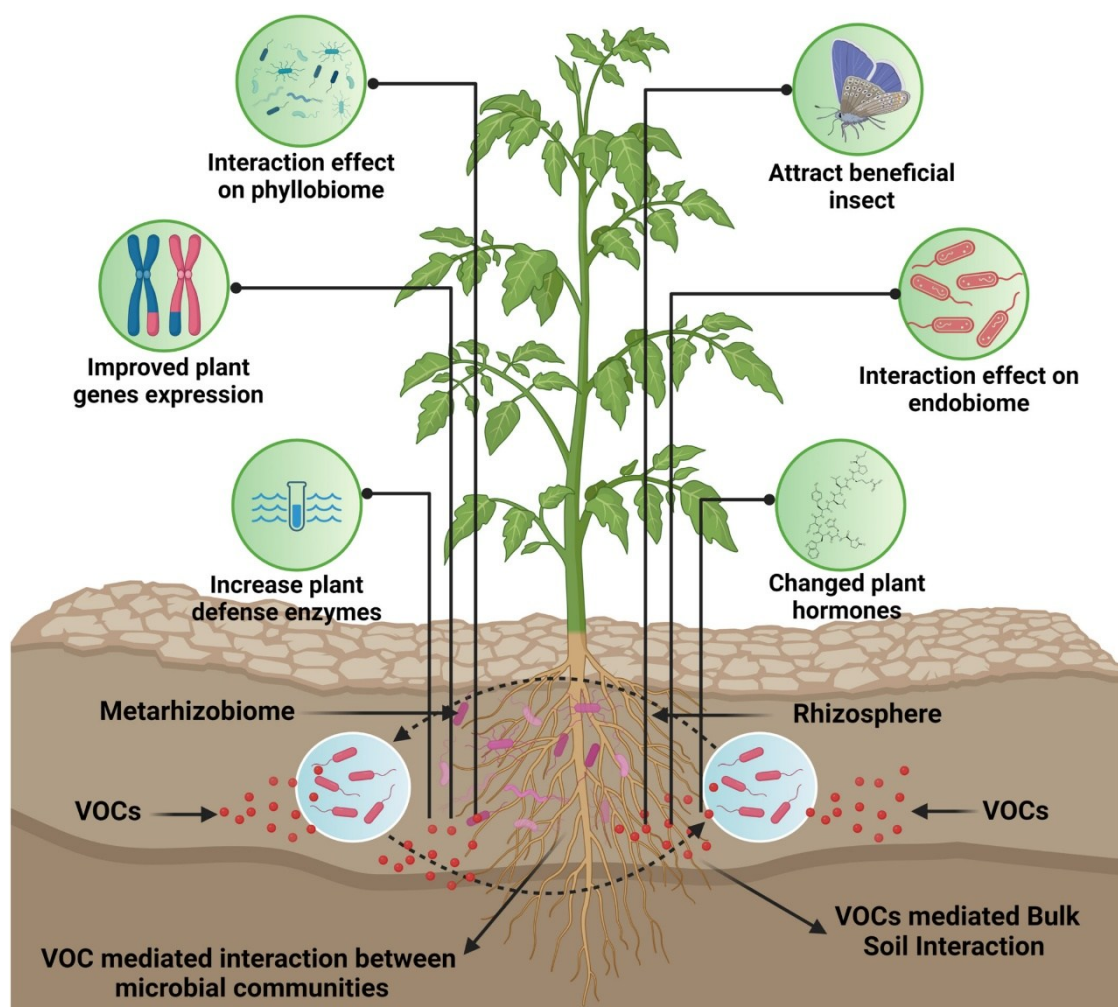


Figure 7: Volatile organic compounds produced by microorganism positively affect plant growth and development. (Ali et al., 2025)

Table 1: Main ecological roles of the principals VOC classes produced by microorganisms.

Compound classes	Microbial source	Biological and functional role in soil	Key references
Terpenes	Bacteria; Fungi	Bacteria-bacteria, bacteria-fungi and bacteria-plant interaction Antibacterial activity	Schulz-Bohm et al., 2017 Ali et al., 2025
Ketones	Bacteria; Fungi	Antifungal activity	Ali et al., 2025 Lu et al., 2025
Benzenoids	Bacteria	Antimicrobial activity Plant growth-promoting activity	Poveda, 2021
Aldehydes	Fungi; Bacteria	Antifungal activity	Morath et al., 2012 Syed-Ab-Rahman et al., 2019
Cyclohexanes	Bacteria	Antifungal activity	Insam and Seewald, 2010
Alcohols	Bacteria; Fungi	Antimicrobial activity Plant-growth-promotion activity	Fincheira and Quiroz, 2026 Hung et al., 2013 Intana et al., 2021
Pyrazines	Bacteria	Antifungal activity Communication Defense	Huang et al., 2025 Bağdat et al., 2025
Phenols	Fungi	Antimicrobial activity Plant growth regulation Nematocidal activity	Werner et al., 2016

Metabarcoding analysis as a tool to study soil and root associated microbiota

Soil health is directly linked to its microbial composition (Djemiel et al., 2022). The presence of specific taxa is directly related to the biogeochemical cycle of major elements, the degradation of soil toxic compounds, plant growth promotion, and soil structure improvement (Djemiel et al., 2022; Schlöter et al., 2018). Moreover, soil microbiota can rapidly change under soil disturbance phenomena, allowing an early assessment of soil health conditions (Djemiel et al., 2022; Griffiths and Philippot, 2013).

Metabarcoding is a technique that uses universal primers to detect all organisms in a specific environment, targeting millions of DNA fragments (Zhang M. et al., 2023). Identifying soil microbial communities through metabarcoding allows to understand soil health conditions, linking taxa composition to its bio-functional role in the ecosystem. For example, this technique allows to identify beneficial soil microorganisms, able to improve plant growth or taxa involved in soil bioremediation, able to degrade toxic compounds (Francioli et al., 2021; Wang Q. et al., 2024). With metabarcoding, it is possible to identify taxa composition in different environmental samples via amplification and high-throughput sequencing of specific regions, known as the barcode marker genes (Semenov, 2021). These regions usually include rRNA gene for Prokaryotes, ITS (internal transcribed spacer) for Fungi, and 18S rRNA gene for most of Eukaryotes (Semenov, 2021). They contain conserved sequences that allow the recognition of taxa belonging to high taxonomic ranks, such as domain and kingdom, and high variable sequences that allow the identification of different taxa. For these reasons, they represent useful marker regions in metabarcoding analysis (Nilsson et al., 2019; Yarza et al., 2014).

Sequencing may target DNA sequences of different length. The first-generation sequencing technology is represented by Sanger sequencing. This technique provides 700-1000 bp-length reads but is expensive and with a low repeatability (Sanger et al., 1977). To overcome the limits of this technique, the Next-generation Sequencing (NGS) technologies have been developed. These methods allow the amplification of short DNA fragments (50-300 bp) with a high level of repeatability and lower costs of application. Among the different NGS techniques, Illumina sequencing is the most commonly adopted because of its suitability for a wide range of applications, high sensitivity and its capacity in producing a large amount of reads *per run*

(Goodwin et al., 2016; Metzker, 2010). In the last decades, the Third-generation sequencing techniques have been developed and their use is spreading. These technologies provide single-molecule long-read sequencing, allowing the sequencing of regions as the whole 16S (Clarke et al., 2009; Goodwin et al., 2016). The main third-generation technologies are PacBio and Oxford nanopore and differ from the previous ones because they may operate without PCR amplification *prior* sequencing, potentially reducing PCR-related biases. PacBio allows the sequencing of 10-25 kb reads and is based on a single-molecule real-time (SMRT) approach (Eid et al., 2009; Goodwin et al., 2016). The Oxford nanopore technology (ONT) is more recent and provides the sequencing of DNA fragments up to 100 kb. The previous technologies have always based their functionality on adding and reading each nucleotide recognized by a specific labelling. On the contrary, ONT provides the recognition of the nucleotides associating to each variation in the electrical current of the instrument. This allows the real-time detection of each nucleotide in native DNA molecules (Clarke et al., 2009; Goodwin et al., 2016). The limits of third-generation sequencing are primarily the need of high-quality extracted DNA, able to provide long reads to be sequenced, the high costs of technologies and the higher errors rate compared to NGS (Bleidorn, 2016; van Dijk et al., 2018). For this reasons, NGS technologies as Illumina are still largely used for metabarcoding.

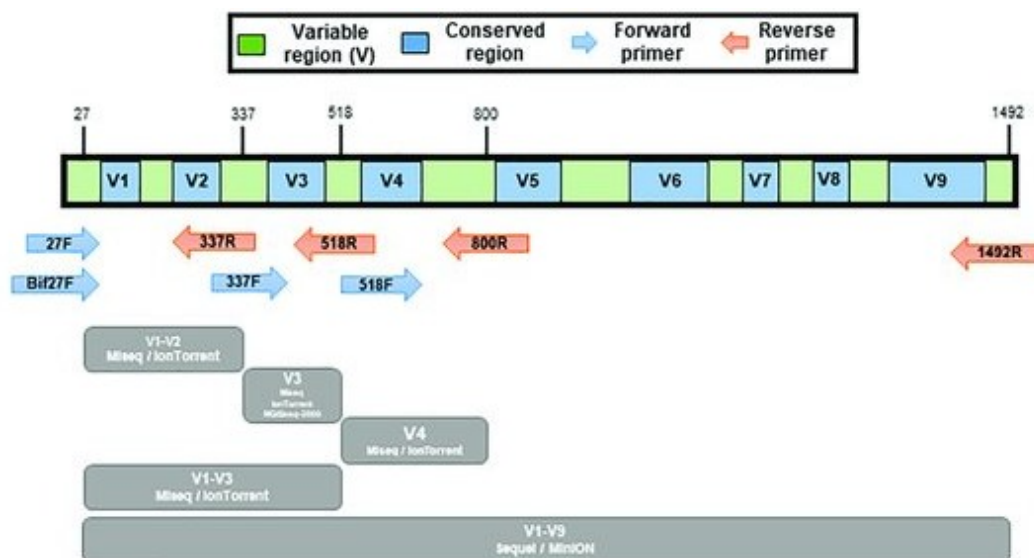


Figure 8: 16S sequence, its regions and the relative primers that can be used for amplification (Park et al., 2011).

The 16S rRNA gene is characterized by different regions (V1-V9) that can be amplified with different primers, thereby targeting different group of Prokaryotes with different specificity (**Fig 8.**, Park et al., 2021).

Usually, the amplification of 16S for prokaryotic communities is performed selecting V4 region or amplifying the whole sequence of the 16S. The V4 region is often selected because provides a good accuracy in taxa discrimination and the relative low cost for sequencing compared to other regions (Zhang et al., 2023) and can be amplified by using primers 515F and 806R. Often, the V4 region is also amplified with the V3, using primers 341F and 806R as forward and reverse, respectively. These primers are optimal to detect prokaryotic communities in soil samples (Klindworth et al., 2013; Lee et al., 2023). The whole 16S amplification is performed by using primers 27F and 1492R. The amplification of all the 16S sequence, that can be achieved with the Third-generation sequencing techniques, offers a good overview of complex systems such as the soil environment, characterized by a high taxa diversity, but is obviously more expensive (Boutsika et al., 2024; Pascoal et al., 2024). Thus, the choice of the region to be amplified should consider all these different aspects.

ITS sequence is composed of ITS1 and ITS2 regions (**Fig. 9**). Amplification may target only one region or both. Amplifying ITS1 with primers ITS1F and ITS2 provides good accuracy in targeting soil fungi while avoiding plant DNA amplification, whereas amplifying ITS2 region can be achieved using primers fITS7 and ITS4 as forward and reverse, respectively (White et al., 1990). the whole sequence with ITS1F and ITS4 often offers good accuracy at the species level (White et al., 1990). Thus, again, the choice should consider these different aspects.

Concerning AMF, the amplification of the ITS region is not optimal. In fact, AMF are characterized by high inter-genomic variability, with several copies of divergent sequences in the same organism. The primers for ITS sequencing are developed to better address the amplification of other fungi DNA (Stockinger et al., 2010). It has been demonstrated that amplification of 18S leads to a better resolution of AMF diversity compared to the ITS region, and it is, therefore, preferred (Guillen-Otero et al., 2023). Sato et al. (2005) developed a new set of primers, named AMV4.5NF and AMDGR for the 18S region, which are currently useful tools for targeting only AMF sequences.

Apart from primer choice, another important step to achieve better specificity in microbial identification is selecting the database for taxonomic classification.

Databases for taxonomic classification

Bacteria are generally taxonomically classified using two alternative databases, *i.e.*, the Silva (Quast et al., 2013) and the Greengenes databases (DeSantis et al., 2006). Silva database contains a collection of 16S, 18S and 23S sequences and it is used to identify taxa belonging to Archaea, Bacteria and Eukarya domains. The Greengenes database contains only 16S sequences, and it is not updated as frequently as Silva. According to Lyte et al. (2025), the last update of the database

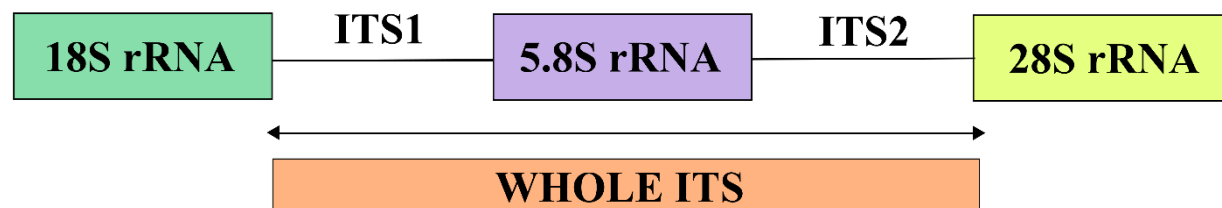


Figure 9: ITS regions.

seems to date to 2013. Therefore, to address the identification of Prokaryotes and Eukaryotes, it is suggested to use the Silva database (Park et al., 2021). Among the different databases that can be used, it is important to mention the Ribosomal Database Project (RDP), that contains small subunit (SSU) rRNA sequences for bacteria and large subunit (LSU) rRNA sequences for fungi (Cole et al., 2014). The major limit of this database is that most of the sequences derive from PCR products and are short and incomplete, especially in proximity of the 3' and the 5' ends. Due to the short reads, the taxonomic classification at low taxonomic levels, such as species and strains, is not accurate. For these reasons, this database is not often used as Silva and Greengenes (Almeida et al., 2018).

Concerning the common taxonomic identification of fungi, the most used database is UNITE (Nilsson et al., 2019). UNITE database contains ITS sequences and targets specifically fungi. Anyway, as previously mentioned, AMF are not optimally identified using ITS sequences. To fill this gap, from 2010 a new database based on 18S sequences and specific for AMF has been developed (Opik et al., 2010). The MaarjAM database is mostly composed of sequences of the small subunit rRNA gene and allows the identification of virtual taxa (VT). Sequences with percentage of identity higher of 97% are grouped in operational units called virtual taxa and each VT represents a species or a phylogenetically high cohesive group (Opik et al., 2010).

Tools for microbial taxonomic assignment

Among the different techniques and tools that can be used to identify taxa associated with the study conditions, one is represented by the QIIME2 framework (Bolyen et al., 2019), which can be installed in miniconda3 environment. Miniconda3 represents a lighter version of Anaconda and allows you to install QIIME2 rapidly and efficiently (<https://anaconda.org/>). Anaconda is an open-source distribution of the Python and R programming languages, specifically designed for data science, machine learning, and artificial intelligence applications. It simplifies package management and deployment and it is particularly useful in bioinformatics for handling complex dependencies and environment configurations.

Briefly, the QIIME2 pipeline that has been used in the different research activities within the PhD provided read import, quality check, denoising (trimming sequences at specified, lengths

based on quality), ASV/OTU calling, and alignment to a reference database for taxonomy assignment.

Taxonomic assignment can follow two distinct pathways: the production of Operational Taxonomic Units (OTUs) or the production of Amplicon Sequence Variants (ASVs). OTUs are produced through a reads-clustering process, grouping reads with at least 97% similarity. OTUs that are similar for less than the 3% represent the same taxa (Callahan et al., 2017; Joos et al., 2020). Amplicon Sequence Variants production is based on *de-novo* reconstruction of reads after correcting sequencing errors, identifying variations that occur due to biological differences among samples or sequencing errors. This method is based on the fact that biological variations are most frequent than sequencing errors and are more likely to be repeatedly observed than the error-containing sequences (Callahan et al., 2017). Nowadays, ASVs are more commonly used than OTUs because they can detect small variations in whole sequences, distinguishing sequence variations even by one nucleotide (Callahan et al., 2017). This approach allows for better resolution for bacterial communities, where lower taxonomic levels, such as strains, can be distinguished based on very small variations. In the PhD activities, by using QIIME2 pipeline, we generated ASV tables, consisting of matrices quantifying the occurrence of each ASV across a set of biological samples. Each row corresponds to a unique taxon, and each column represents a sample. The obtained ASV table can be used to assess differences in taxa composition and abundance among the different study conditions.

The previously described steps allow to identify differences in the taxa composition across different samples. These differences are mainly evaluated through alpha and beta diversity. Alpha diversity measures the richness and evenness of taxa within a sample, while beta diversity evaluates the differences in overall community composition among different conditions (Cassol et al., 2025, **Fig. 10**).

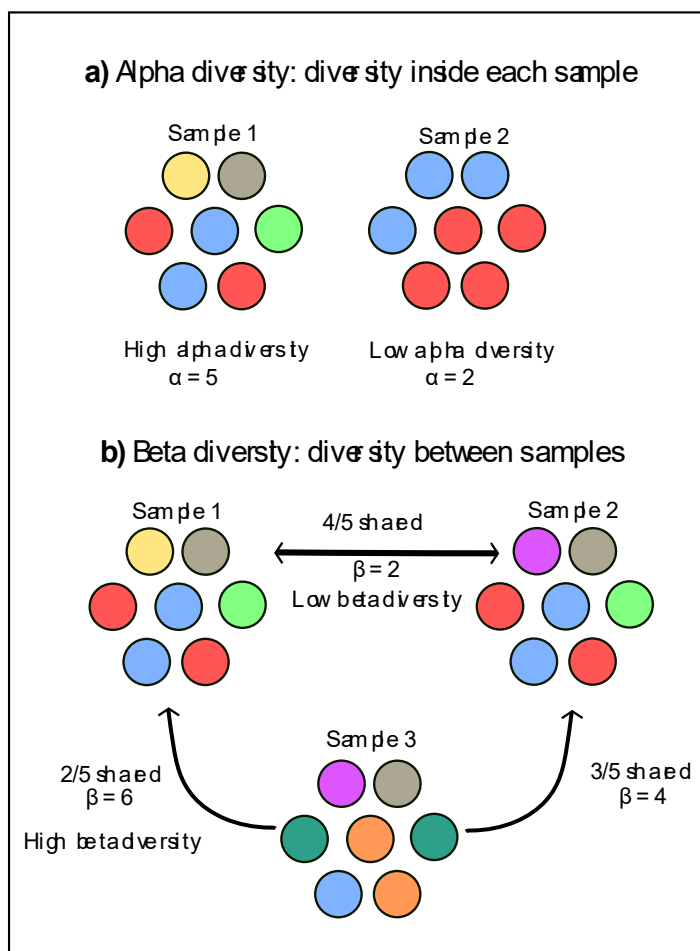


Figure 10: Alpha (a) and beta (b) diversity explanation.

There are several ways to evaluate taxa composition among different environmental conditions. Among them, the Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC) identifies differentially abundant taxa by correcting for sampling bias and compositional effects, providing more accurate estimates of taxa relative abundances and strengthening the reliability of observed differences across samples (Lin and Peddada, 2020). Basically, it serves to remove the bias of having a different number of reads associated to each analyzed sample, due to differences in sequencing depth. It works using a statistical model (usually based on a linear regression)

with bias correction, removing the sampling fraction bias from each sample and providing a common scale to evaluate taxon differential abundance.

Another method that can be used to discriminate taxa among different conditions is the Linear Discriminant Analysis Effect Size (LEfSe), that is constituted on three different step: i) first, significant variations in the different condition of taxa abundance are evaluated through statistical assessment, ii) then, this variations are evaluated in the sub-classes of the different conditions, and iii) finally, a linear discriminant analysis is performed to evaluate how each taxa may discriminate a specific study condition, determining its effect size (Segata et al., 2011).

Moreover, it is possible to evaluate specific taxa associations with the study conditions using DeSeq2. This statistical method was originally built for RNA-seq analysis but it now widely

adopted also in microbiome assessment studies. The method is based on a first normalization of row count data, removing the bias of differential sequencing depth. Then, the dispersion for each sample is calculated to evaluate the variability in taxa composition among samples within the same condition and a sub-sequent normalization of these dispersions (shrinkage) is made. The next step is the differential abundance testing, in which the differences in taxa abundance among conditions are returned as log 2 fold change values, where positive and negative values reflect an increase or a decrease in the taxa abundance. With the log2 fold change also a p -value for each variation is provided. A further step of this method is the calculation of the adjusted p -value, to overcome the errors due to multiple simultaneous comparisons among taxa (Love et al., 2014).

These different methods offer different advantages. ANCOM-BC is more robust in taxa abundance evaluation and, as well as DeSeq2, it provides a more accurate bias correcting process, while LEfSe offers a higher discriminatory power among the different conditions. Moreover, DeSeq2 is recommended for robust fold-change testing. Thus, each method application highly depends on the adopted experimental design and on the raw data composition.

Aim of the Thesis

During the PhD, I worked in the Interaction Laboratory group at CNR-IPSP. The group is specialized in plant-microorganism interactions to promote plant growth under stress conditions, with a particular focus on the application of AMF as beneficial microorganisms for sustainable plant production. This thesis aims to investigate the relationships among soil microbial communities and different types of crops using metabarcoding approaches to promote plant growth in arid, semi-arid, and degraded environments. The thesis is divided in four chapters, representing diverse in field and in pot experiments aimed to investigate the impact of different extreme environmental conditions on diverse agricultural and urban ecosystems.

Chapter I. To evaluate the role of microbial-based solutions in improving plant resistance to drought, a pepper variety (*Capsicum annum* “Locale di Senise”) was used in an open field trial in Metaponto di Bernalda, Southern Italy, a typical Mediterranean habitat strongly affected by diverse environmental threats. Plant response to drought was investigated by applying different irrigation levels in combination with AMF inoculation. Soil microbial communities variations driven both by drought and AMF inoculation were also analyzed. This study suggests that optimization of irrigation and beneficial plant–microbe interactions improves pepper growth under water limitation conditions.

Chapter II. This second activity was focused on another important group of beneficial soil microorganisms, *i.e.*, the PGPB, particularly within the framework of a collaboration with the group of Bacem Mnsari, full Professor at Centre of Biotechnology of Borj Cedria (Tunisia) and with a Tunisian PhD student during her stage at CNR-IPSP. To assess the role of PGPB in improving plant resistance to drought, a two-year field study was conducted in Mateur, Tunisia, a well-known drought-prone environment. One strain of *Rhizobium laguerreae* and two strains of *Erwinia* sp. were inoculated in two *Pisum sativum* varieties (Douce de Provence and Merveille de Kelvedon). The first objective of this work was to assess PGPB beneficial effect on biometric traits and yield of the two pea varieties. A further aim of the study was to investigate how these bacterial inoculations influenced pea-associated microbial communities. Results allowed us to investigate each variety responses to microbial inoculation. This highlighted the potential of

microbial consortia to enhance pea growth and productivity by exploiting synergistic interactions between specific bacteria and AMF, thereby supporting sustainable agricultural practices.

Chapter III. This chapter explores the role of intercropping as a sustainable agricultural practice. Among the sustainable agricultural practices that can be used, intercropping represents an economic, ecological, and environmentally friendly practice. Legume-based intercropping systems provide additional benefits thanks to the ability of legume-associated rhizobia in fixing N₂, reducing the dependence on chemical fertilizers and the related negative impact on the environment. To investigate the positive effect of legume-based intercropping in arid and semi-arid regions, a study was conducted by the group of Prof. Bacem Mnsari on *Brassica napus*. This crop cannot form symbiotic relationship with N-fixing bacteria; therefore, its production strongly relies on chemical fertilizers. The study was conducted in Mateur, Tunisia, using *B. napus* as monoculture, as well as *B. napus* intercropped with *Hedysarum coronarium* and with *Medicago sativa*, two forage legumes. The primary objective of this work was to evaluate the impact of the two intercropping systems on the agronomic performance of *B. napus* over two growing seasons. Moreover, the study investigated how intercropping influenced rhizosphere and root-associated bacterial communities, with the objective of determining whether these practices are able to simultaneously enhance crop productivity and soil health. Thanks to the stage of a second PhD student hosted at the CNR-IPSP, I had the possibility to work on the analyses of the shared data. This work allowed us to understand that *B. napus* production strongly depends on the associated legume species, especially in water deficit conditions, and that this practice shapes *B. napus* root-associated bacterial communities, suggesting a differential microbial recruitment mediated by the presence of a specific legume.

Chapter IV. This last chapter is dedicated to the investigation of the role of AMF in restoring soil health in urban environments. Urban soils are constantly subjected to degradation processes, leading to a consequent loss of biodiversity and an increasing negative impact on the health of all organisms. One of the newest approaches in evaluating soil health is based on the study of microbial communities and the identification of VOCs, both of anthropogenic and microbial production, as markers of soil health status. Our study was conducted in a mesocosm experiment using de-sealed soil from an urban site as substrate with AMF inoculation, along with two cover plant species, *Cynara cardunculus* L. and *Trifolium repens* L. This study investigated the ability

of AMF to colonize plants in reused soil after de-sealing, while also evaluating the potential of AMF inoculation combined with cover plants to contribute to the restoration of soil health through the enhancement of pollutant degradation by autoxidation of Anthropogenic Volatile Organic Compounds (AVOCs). Furthermore, the capacity of native AMF to survive, recover, and colonize plant roots after soil de-sealing was examined as an additional mechanism supporting soil health restoration. Results showed that the inoculation of the de-sealed, degraded soil with a commercial AM fungal inoculum, combined with cover plants, contributed to the restoration of soil health, partly through enhanced bacterial diversity. The establishment of complex microbial consortia supported AVOCs degradation, underscoring the bioremediation potential of this strategy in urban soils.

Chapter I

The first chapter of this thesis is the result of a work published on Plant and Soil in 2025. The objective of this study was to evaluate the effect of AMF inoculation under water-limited conditions designed to simulate drought stress in a drought-sensitive crop such as pepper (*Capsicum annuum*).

The analysis focused on pepper eco-physiological parameters, as well as on its qualitative and quantitative traits, as well as on the impact of AMF inoculation on the native soil microbial community. The open-field experiment took place at the “Agenzia Lucana di Sviluppo e di Innovazione in Agricoltura” (ALSIA) research center “Azienda Pantanello”, in Metaponto di Bernalda, Matera, Italy.

The results of this study provided novel insights into the impact of applying an AM fungal inoculum in the field, highlighting the complex relationship among plants, AMF, and the natural microbiota of the soil under stressed conditions.

Interactions between an arbuscular mycorrhizal inoculum and the root-associated microbiome in shaping the response of *Capsicum annuum* “Locale di Senise” to different irrigation levels

Alice Calvo · Thomas Reitz · Fabiano Sillo · Vincenzo Montesano · Eva Cañizares · Elisa Zampieri · Roghayyeh Mahmoudi · Gholamreza Gohari · Walter Chitarra · Luca Giovannini · Adriano Conte · Carmelo Mennone · Gianniantonio Petruzzelli · Mauro Centritto · Miguel González-Guzmán · Vicent Arbona · Vasileios Fotopoulos · Raffaella Balestrini 



Received: 6 April 2024 / Accepted: 10 June 2024

© The Author(s) 2024

Abstract

Background and aims The use of root-associated microorganisms emerges as a sustainable tool to enhance crop tolerance and productivity under climate change, particularly in drought-affected areas. Here, the impact of an inoculum based on arbuscular mycorrhizal fungi (AMF) was evaluated on pepper

Responsible Editor: Gaowen Yang.

Alice Calvo and Thomas Reitz contributed equally to this work.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11104-024-06806-4>.

A. Calvo · F. Sillo · E. Zampieri · L. Giovannini · M. Centritto
Institute for Sustainable Plant Protection (IPSP), National Research Council of Italy, Turin, Italy

A. Calvo
Department of Agriculture and Desertification Research Centre, University of Sassari, Sassari, Italy

T. Reitz
Department of Soil Ecology, Helmholtz Centre for Environmental Research GmbH – UFZ, Halle, Germany

T. Reitz
German Centre for Integrative Biodiversity Research (iDiv), Halle-Jena-Leipzig, Germany

(*Capsicum annuum* L.) cultivation at varying water irrigation treatments (well-watered, reduced irrigation and rain-fed) under open-field conditions.

Methods Agronomic and ecophysiological parameters, as well as biochemical analyses on stress markers and phytohormones in leaves and on fruit quality traits, were evaluated, along with the shifts in soil and root-associated microbial communities.

Results Rain-fed water treatment caused reduced

fruit sizes, while no differences were detected among well-watered and reduced irrigation. Reduced irrigation did not cause a reduction in stomatal conductance. The highest AM fungal colonization rates were observed under reduced irrigation, and the enhanced flavonoid content and reduced oxidative stress markers in AMF-inoculated plants suggested a synergistic effect of AM fungal inoculation in boosting plant tolerance against stress. A shift in microbial community

V. Montesano · A. Conte
Institute for Sustainable Plant Protection, National Research Council of Italy, Metaponto di Bernalda, MT, Italy

E. Cañizares · M. González-Guzmán · V. Arbona
Departament de Biologia, Bioquímica i Ciències Naturals, Universitat Jaume I, Castelló de La Plana, Spain

R. Mahmoudi · G. Gohari · V. Fotopoulos
Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, Limassol, Cyprus

composition in the different irrigation treatments, associated with different enzymatic activity, highlighted the potential role of microbial dynamics in plant stress response under water-limited conditions.

Conclusion The study suggests that a reduced irrigation comes along with beneficial impacts on pepper root associated microbes, while not impairing crop performance and yields, indicating a potential of saving water. All together, our results imply that optimization of irrigation and beneficial plant–microbe interactions, such as AM fungal symbiosis, can improve pepper physiological and productivity features under climate change.

Keywords Pepper · AM symbiosis · AM fungal inoculation · Drought · Irrigation · Tolerance

Introduction

Drought is one of the most impactful abiotic stresses, significantly affecting crop yield and production, thereby posing risks to global food security (Food Insecurity 2022). In addition, as a consequence of global climate change, drought is expected to intensify in the near future, representing a further significant threat to agricultural areas increasing the risks of crop production decline. This underscores the urgent need to explore novel sustainable practices aimed at improving water use efficiency in agroecosystems (Bhattacharyya et al. 2021). Over the last decades, plant responses occurring during drought and recovery phases have been largely studied at many levels, from cell signaling to the whole ecophysiological

traits for both herbaceous and woody crops (Zia et al. 2021). However, it is worth noting that plants share their environment with several soil microbes that live associated to the roots and in the rhizosphere, establishing with their hosts a complex network of exchanges crucial for the maintenance of plant health (Berendsen et al. 2012). These intimate associations between plants and their inhabiting microbes allow mutual responses with the surrounding environment *e.g.*, tolerance to abiotic stresses and improved nutrient uptake (Sandrini et al. 2022; Zhang et al. 2021). Beyond the well-known detrimental effects of drought on plants, including reductions in photosynthetic and stomatal conductance rates as a consequence of hydraulic impairment (Haworth et al. 2021), as well as metabolic (*e.g.*, abscisic acid – ABA production and oxidative burst by reactive oxygen species – ROS) (Marino et al. 2017) and nutritional imbalances, stressed plants, in turn, activate responses to cope with stress (*e.g.*, osmoprotectants and antioxidant accumulations) (Ahluwalia et al. 2021; Mahmood et al. 2021; Sandrini et al. 2022; Tang et al. 2023). These responses span from molecular signaling to phenotypic adaptation characteristics (Naseem et al. 2018; Nerva et al. 2023). Among these traits, root traits play key roles in protecting plants from desiccation. This involves root apparatus growth to explore a larger soil volume and the release of exudates, whose composition has been reported to change during stress, influencing the shifting of rhizospheric microbiome structure and dynamics (de Vries et al. 2020). Drought alters both the quantity and quality of root exudates, exerting selective pressure on favorable rhizospheric microbiomes capable of mitigating drought-induced damages in stressed plants (Williams and de Vries 2020). Beneficial soil microorganisms have the capacity to mitigate the effects of water deficit in plants by influencing several processes (Fadiji et al. 2022) *e.g.*, via osmolyte, antioxidant and phytohormone production (Brunetti et al. 2021). Beneficial soil bacteria can enhance soil micropores through the secretion of various compounds, such as exopolysaccharides. This phenomenon results in increased water retention in the soil, thereby fostering plant resilience against water deficit conditions (Philippot et al. 2024). Soil microbiome can have a positive effect also on nutrient uptake, facilitating the availability of elements that are not accessible in other forms or producing molecules able

W. Chitarra
Council for Agricultural Research and Economics, Research Centre for
Viticulture and Enology (CREA-VE), 31015 Conegliano, TV, Italy

C. Mennone
Azienda Pantanello, ALSIA Research Center Metapontum Agrobios, Meta-
ponto di Bernalda, MT, Italy

G. Petruzzelli
Institute of Research on Terrestrial Ecosystems (IRET), National Research
Council, Pisa, Italy

R. Balestrini (✉)
Institute of Biosciences and Bioresources (IBBR),
National Research Council, Bari, Italy e-mail:
raffaellamaria.balestrini@cnr.it

to mitigate water stress (Ali et al. 2022; Caddell et al. 2023; Philippot et al. 2024). In addition to soil bacteria, numerous studies have demonstrated that Arbuscular Mycorrhizal Fungi (AMF) can improve plant growth as they facilitate the absorption of nutrients and water, particularly in challenging environmental conditions such as drought (Augé et al. 2015). The fungal hyphae of AMF have the capability to explore soil pores that are beyond the reach of root hairs, thus enhancing the plant water uptake and ultimately boosting crop productivity under water deficit conditions (Chitarra et al. 2016; Kakouridis et al. 2022). Currently, both AMF and beneficial soil bacteria are representing promising bio-based tools to improve plant tolerance to stresses (Abdelkhalik et al. 2023; Sandrini et al. 2022; Zhang et al. 2022a).

Pepper (*Capsicum annuum* L.), a member of the Solanaceae family, is a globally grown crop (typically annual cycle) with an estimated production exceeding 40 million tons per year and cultivated over 3.6 million hectares, making it the second most cultivated vegetable after tomato (FAOSTAT 2021; Olmstead et al. 2008). Additionally, pepper berries are known for their high nutritional value as a source of antioxidant and nutraceutical chemicals (Soare et al. 2017). Due to a wide transpiring leaf surface and high stomatal conductance, as well as to the high-water requirements needed during its growing period, pepper is considered as a drought susceptible crop (Alvino et al. 1994; Delfine et al. 2001). The application of different AM fungal inocula has proved to alleviate water and salt stresses by triggering various molecular (e.g., aquaporin and dehydrin genes) and biochemical (e.g., antioxidant enzymes) pathways in several crops (Chitarra et al. 2016), including different pepper cultivars, i.e., the commercial Sera Demre 8 and the Polish sweet pepper hybrid Roberta F1 (Güneş et al. 2023; Nurzyńska-Wierdak et al. 2021). Under water deficit conditions, an increase in root width, length, and weight due to the application of AM fungal inocula has been observed in pepper plants, enabling them to deeply explore larger volumes of soil (Tang et al. 2023; Türkmen et al. 2008).

Interestingly, it has been already reported that natural soil microbiota seems to be affected by AM fungal inoculation (Jing et al. 2022; Nerva et al. 2022). A study on *Cucurbita pepo* L. rhizosphere has recently revealed a higher presence of plant growth-promoting microorganisms in presence of a *Funneliformis mosseae* inoculation, compared to not-inoculated plants (Wang et al. 2022). A similar scenario was observed in *C. annuum* rhizosphere after *F. mosseae* inoculation (Tang et al. 2023). Hence, it is evident that a deeper understanding of plant–microbe and microbe–microbe interactions during water deficit, with a specific emphasis on the rhizosphere compartment, is essential. This knowledge

is pivotal for enhancing crop tolerance to drought (Santos-Medellín et al. 2021).

Here, by using *C. annuum* “Locale di Senise” plants as a typical crop of Mediterranean area, a study in small plots under open-field condition was conducted, providing a realistic representation of production environments. The study aimed to investigate the impact of AM fungal inoculation on pepper plants, subjected to different irrigation treatments considering plant physio-chemical responses and AM fungal interaction with the natural soil microorganisms.

Materials and methods

Experimental design

The open-field experiment took place at the Lucan Agency of Innovation and Development in Agriculture (ALSIA) research center “Azienda Pantanello”, in Metaponto di Bernalda, Southern Italy (40°23′031.4″ N, 16°47′010.9″ E). Early growth stage pepper plants (cv “Locale di Senise”, 3–4 expanded leaves) were transplanted on June 18, 2022. For approximately 40 days after transplantation, till the plant architecture was completely formed, water was supplied with amounts equivalent to 100% of the effective crop evapotranspiration (ETc). After that stage (from July 22, 2022) a plot was kept in well-watered conditions (100% of ETc—R1), a second plot was kept under reduced water conditions (75% of ETc—R2), while the third plot was kept in rain-fed condition (R3). The daily ETc was calculated according to the formula $ET_c = ET_0 \times K_c$, where ET_0 is the reference evapotranspiration according to Penman–Monteith equation (Allen et al. 1998) and K_c is the crop coefficient, based on recommendations for bell pepper production and adjusted for the environmental conditions (Ávila-Dávila et al. 2021; Rodríguez Padrón et al. 2015). The water supply was monitored by three MultiJet water meters RMM DN40 (Raphael Valves Industries Ltd, Isr.).

From transplantation to end of the experiment, well-watered parcels (R1) received 50.2 m³ of water, parcels subjected to reduced irrigation (R2) received 35.3 m³ of water, while the R3 treatment received 9.5 m³ of rainfall water. Well-watered parcels and parcels subjected to reduced irrigation received both irrigation and rainfall water. Meteorological data from a weather station positioned at the Azienda Agricola Pantanello were recorded to follow the climatic conditions during the crop growth (Table S1 and Fig. S1). Irrigation was performed using an in-line drip irrigation system (1.3 L/h; 16 mm inside diameter; 20 cm spacing) designed to ensure full water coverage and uniform distribution.

Each single main plot was subsequently divided into two sub-plots, assigned to two different inoculation treatments (I and NI) of AM fungal mixed inoculum (INOQ GmbH, Germany), thus resulting in the following six treatments: IR1, IR2, IR3, NIR1, NIR2 and NIR3. Each single sub-plot was replicated three times (I, II and III) for a total of 18 parcels (six for each irrigation level) spaced 1 m apart. Each parcel consisted of 54 plants, arranged in 5 lines, 4.8 m long for a total of 19.5 square meters (Fig. S2).

For the treatments involving AM fungal inoculation (IR1, IR2 and IR3), a commercial AM fungal inoculum was applied during the transplantation. In detail, each plant was dipped in a water suspension of the AM fungal inoculum provided as wettable powder at recommended doses (INOQ ADVANTAGE; INOQ GmbH, Schnega, Germany). The inoculum contained *Rhizoglyphus irregularis*, *Funneliformis mosseae*, *Funneliformis geosporum* with a minimum of 150,000 mycorrhiza units, i.e., propagules composed by spores, hyphae, and root residues (per gram). Not-inoculated treatments were named NIR1, NIR2 and NIR3. Weeding was carried out by hand and integrated production agronomic management for sweet pepper was applied. In detail, the total amount of applied NPK fertilization, corresponding to 70, 45 and 160 kg/ha, respectively, was split into five rates during the crop cycle: 30% at transplanting (as ammonium sulfate and urea phosphate), 10% at 30 and 50 days after transplanting (as urea phosphate, ammonium nitrate, and calcium nitrate for fertilization), 30% at full fruit set and 20% during fruit development (as 20/20/20 fertilizer and potassium nitrate). Plant protection was carried out according to the standard cultivation protocols of the Basilicata Region (Italy) for integrated production, using different commercial products, applied at different times, at the recommended doses on the product labels: oil-based suspension of *Beauveria bassiana*, suspension concentrate of *Bacillus thuringiensis* subsp. *kurstaki*, suspension concentrate of Azadirachtin, and water-dispersible granules of *Bacillus amyloliquefaciens*.

Sampling procedure

At the beginning of the experiment, after transplanting, a representative soil sample for each parcel was collected. Samples collected from the same inoculation treatment were pooled (one for I and one for NI treatment) and were analyzed for chemical-physical properties. During and at the end of the experiment, two soil sampling time points were established, in August and in October 2022, respectively. For chemical-physical analysis, three replicates for each treatment were pooled to obtain 12 soil samples (six collected in August and six collected in October). Thirty-six bulk soils samples (approximately 800 g per sample), collected twice (August and October 2022), were analyzed for soil enzymatic activity, soil moisture content and assessment of fungal and bacterial community through metabarcoding. In October, at the end of the experiment, roots were sampled for both assessment of mycorrhizal colonization and metabarcoding analysis (three replicates for each treatment, for a total of 18 samples). Leaves (three replicates for each treatment) were collected in August to perform biochemical markers and phytohormones analysis. Fruits (three replicates for each treatment) were collected during both sampling times to perform antioxidant and nutritional analysis.

Soil moisture and extracellular enzyme activities

Moisture content was evaluated using a halogen moisture analyzer on the 36 bulk soil samples (Mettler Toledo, Gießen, Germany). A modified fluorometric assay of Sinsabaugh et al. (2003) was used to determine extracellular enzyme activities of the 36 soil samples. The activity potentials of six hydrolytic soil enzymes, involved in the degradation and acquisition of carbon, nitrogen, phosphorous, and sulfur (β -glucosidase, xylosidase and cellobiohydrolase, N-acetylglucosaminidase, acid phosphatase and sulfatase), were measured as turnover rate of 4-methylumbelliferon (MUF)-coupled substrates. The amount of released MUF was directly related to enzymatic activity potentials. A comparable high concentration

of 300 μM was used for all substrates, to prevent an underestimation of activity due to substrate limitation. For each sample, a black 96-well microplate was used. The plates contained the six enzyme substrates, MUF dilutions to calculate quench and extinction coefficients (1.25 and 2.5 μM), as well as substrate and soil suspension controls. Approximately 250 mg of fresh soil was suspended in 50 ml of 50 μM acetate buffer (pH 5) and sonicated for 5 min to break up soil aggregates. Afterwards, the soil suspensions were transferred to the prepared microplates and incubated at 25 °C for 60 min. After the addition of 30 μl 1 M NaOH to stop the enzymatic reactions, fluorescence was measured for eight replicates using a Tecan Infinite 200 PRO plate reader (Tecan Group, Männedorf, Switzerland) at 360 nm excitation and 465 nm emission wavelengths. Enzyme activities were calculated as turnover rate of substrate in nmol *per* gram dry soil and hour (nmol/ g_{soil} h) (German et al. 2011).

Ecophysiological measurements and agronomic parameters

During the phenological stage corresponding to ‘visible pepper berry’, non-destructive measurement was performed using a steady-state Licor LI-600 Porometer/Fluorometer apparatus (LI-COR Biosciences, Lincoln, NE, United States) to assess stomatal conductance (g_s). The measurements were taken on five randomly selected plants for each parcel, measured on the newly fully expanded leaves, in sunny days at noon (between 10:00 and 12:00). At the end of the experiment, ten randomly selected plants from each parcel were selected to determine: plant height (cm), fruit dry matter (g/100 g), fruit length (cm), width (cm) and thickness (mm), fruit number (n) and weight

(g) *per* plant. Yield (t/ha) was evaluated by summing up the two fruit harvests, in August and October.

Assessment of mycorrhizal colonization in roots

Collected roots were decolored with KOH 10% for 30 min at 80 °C, then stained with 0.1% cotton blue in lactic acid overnight and 1-cm-long root portions were used. Fifty-four slides (three slides for replicate of each treatment, for a total of 54 slides) were prepared and analyzed according to the Trouvelot system (Trouvelot 1986) to evaluate the frequency

of mycorrhization (F%), the intensity of mycorrhizal colonization in the root system (M%) and the arbuscule abundance in the whole root system (A%).

Biochemical markers and phytohormones quantification in leaves

A total of 17 samples of leaves, corresponding to three replicates for each of the six treatments, except for the replicate III of NIR3, were used to perform the biochemical analyses. The evaluated biochemical markers included malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and proline. Lipid peroxidation was determined in leaves from the measurement of MDA content resulting from the thiobarbituric acid (TBA) reaction (Christou et al. 2013; Heath and Packer 1968) using an extinction coefficient of 155 $\text{mM}^{-1} \text{cm}^{-1}$. Hydrogen peroxide content was quantified using the KI method (Velikova et al. 2000), while free proline levels were determined using the ninhydrin reaction (Bates et al. 1973). Concerning phytohormones, the concentration of abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), indole-3-acetic acid (IAA) and 12-oxophytodienoic acid (OPDA), was determined by targeted LC/MS approach as previously reported (De Ollas et al. 2021). Briefly, prior the extraction, samples were spiked with 50 ng of the following surrogate analytes to correct for analyte losses and potential analytical drift in the mass spectrometer ($\text{ABA-}^2\text{H}_6$, $\text{IAA-}^2\text{H}_5$, dihydrojasmonic acid, $\text{SA-}^{13}\text{C}_2$). Extraction was carried out in 1 mL ultrapure water for 10 min in a ball mill at room temperature using 2 mm glass beads. Homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C and supernatants recovered and partitioned twice against an equal volume of diethyl ether after adjusting pH to 3.0 with 30% acetic acid. The combined organic layers were evaporated under vacuum in a centrifuge concentrator (Jouan, Saint Germaine Cedex, France) and the dry residues reconstituted in 0.5 mL of a 10% aqueous methanol solution. Prior to injection, extracts were filtered through 0.20 μm PTFE syringe membrane filters and filtrates recovered in chromatography amber glass vials. Samples were analyzed by tandem LC/MS in an Acquity SDS UPLC system (Waters Corp., USA) coupled to a TQS triple quadrupole mass spectrometer (Micromass Ltd., UK) through an electrospray ionization source. Separations were carried out on a C18 column (Luna Omega Polar C18,

50 × 2.1 mm, 1.6 µm particle size, Phenomenex, USA) using a linear gradient of ultrapure acetonitrile and water, both supplemented with formic acid to a 0.1% (V/V) concentration, at a constant flow rate of 0.3 mL min⁻¹. During analyses, column temperature was maintained at 40 °C and samples at 10 °C to slow down degradation. Plant hormones were detected in negative electrospray mode following their specific precursor-to-product ion transitions and quantified using an external calibration curve with standard samples of known amount.

Antioxidant and nutritional analysis on pepper fruits

A total of 18 fruit samples from the six experimental treatments, with three replicates *per* treatment, were chopped and analyzed for antioxidant compounds and nutritional content. For antioxidant compound analysis, about 500 mg (fresh weight) *per* sample were used. Antioxidant compounds included anthocyanin (mg/100 g FW), ascorbic acid (g/kg FW), flavonoid (mg/100 g FW), phenol (mg/100 g FW) and 2,2-difenil-1-picrylidrazyl (DPPH, radical scavenging activity – RSA%). The anthocyanin content was measured spectrophotometrically according to Giusti and Wrolstad (2001). Ascorbic acid content was evaluated with the oxidation of ascorbic acid by 2,6-dichlorophenol-indophenol sodium salt dihydrate (103,028 Merck, Germany) that, in acidic conditions, combined returned a yellow-orange color according to Suntorn-suk et al. (2002). Total flavonoid content was determined according to the method of Zhishen et al. (1999) with some modifications, and phenol quantification was assessed with Folin-Ciocalteu reagent (Singleton et al. 1999). The DPPH radical scavenging activity was determined according to the method described by Dehghan and Khoshkam (2012). Concerning micronutrients analysis, the remaining fruit samples were collected and oven-dried at 65 °C for 4 days to perform micronutrient analysis. Fruits were analyzed for N%, P%, K%, Ca%, Mg%, Na%, Fe (ppm), Mn (ppm), Cu (ppm), Zn (ppm) and B (ppm) content.

Metabarcoding analysis

Thirty-six soil samples and 18 root samples, corresponding to the six experimental treatments, three replicates each, were used for the assessment of microbial communities via metabarcoding analysis. Each soil sample was homogenized by using bead-

beating tubes provided by the DNeasy PowerSoil Pro Kit (Qiagen) and a Vortex Genie ® 2 (Scientific Industries) for 20 min. Total DNA was isolated from approximately 250 mg of soil for each sample using the same kit and quantified with a Nanodrop 2000 (Thermo Scientific). For metabarcoding analysis, 16S rDNA fragment (for Prokaryotes) and ITS2 region (for fungi) were amplified using the KAPA HiFi DNA Polymerase and primers (515f: GTGYCA GCMGCCGCGGTAA, 806r: GGACTACHVGGG TWTCTAAT for 16S rDNA and ITS4: TCCTCCGCT TATTGATATGC and fITS7: GTGARTCATCGA ATCTTTG for ITS2 region). The PCR-amplification was conducted in triplicates for each sample and target region (40 ng DNA template used *per* PCR reaction). The temperature profile for prokaryotic amplicon PCR was the following: initial denaturation at 95 °C for 3 min, 25 cycles of denaturation at 98 °C for 20 s, annealing at 55 °C for 15 s, and extension at 72 °C for 15 s, followed by the final extension at 72 °C for 5 min. The temperature profile for fungal amplicon PCR was: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 98 °C for 20 s, annealing at 56 °C for 20 s, and extension at 72 °C for 20 s, followed by the final extension at 72 °C for 5 min. Success of PCR was checked with agarose gel electrophoresis. Amplicons from the PCR-triplicates were pooled and purified with AmpPure XP Beads, indexed in an additional PCR (Illumina Nextera XT v2 index primers) and purified again with AmpPure XP Beads. Concentration of indexed and purified PCR-products was determined with NanoDrop ND-8000 spectrophotometer. DNA of fungal and prokaryotic amplicons were equimolarly pooled. Exact concentrations of the final pools were determined with Qubit dsDNA-HS Assay and fragment length and quality were additionally checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The prokaryotic and fungal amplicon pools were combined for Illumina MiSeq paired-end sequencing. Sample libraries and the control library were diluted and denatured following the MiSeq Illumina kit instructions and injected into an Illumina MiSeq flow-cell for paired-end sequencing. Metabarcoding data were analyzed with QIIME 2 (Bolyen et al. 2019). Sequences were trimmed with cutadapt v3.4, denoised through dada2 v2021.8.0, and assembled into Amplicon Sequence Variants (ASVs).

The Greengenes2 2022.10 (McDonald et al. 2023) and UNITE v8-99 (Kõljalg et al. 2013) databases for bacteria and fungi, respectively, were utilized to train Naive Bayes classifier on ASVs (99% identity) sequences for taxonomic assignment. Chloroplast and mitochondrial sequences were removed by filtering from the resulting ASV table with the “*filter-features*” parameter of QIIME 2. The output was elaborated to obtain a relative abundance (%) of each ASV in the total amounts of the entire sample. Raw reads from metabarcoding of soil and root samples were submitted to NCBI SRA under BioProject PRJNA1024920 for bacteria and fungi, respectively. The ASV tables were generated for fungal and bacterial communities detected in soil collected in August, as well as root and soil collected in October. The tables were used as input for Microbiome Analyst (Chong et al. 2020; Dhariwal et al. 2017) for data visualization and statistical assessment. Diversity within samples at feature level (alpha diversity) was calculated using the Chao1 index, while diversity among samples at feature level (beta diversity) was calculated with the Bray–Curtis index and reported in a two-dimensional principal coordinates analysis. Taxon abundance was visualized through stacked barplots at the phylum level. Core microbiome heatmap was generated at phylum level for each ASV table. Taxon abundance was investigated specifically for root bacterial communities, to assess roots-microbiome interactions under water-deficit conditions. A single-factor comparison was performed for each analysis at family level, using DESeq2 method embedded in Microbiome Analyst (Love et al. 2014), considering the different conditions separately (inoculation, irrigation treatment, source and sampling period) and their interaction, to evaluate statistical differences of fungal and bacterial communities associated to each sample.

Statistical analysis

R software (version 4.3.1) was used to perform statistical analysis. Two-way ANOVA was employed to examine the impact of the irrigation treatment, inoculation, and their interaction on agronomic and ecophysiological parameters, biochemical marker content and hormones in pepper leaves, levels of antioxidant compounds and micronutrients in fruits, and data of soil enzyme activity. Post-hoc statistical analysis was conducted using the Tukey Honestly

Significant Difference (HSD) test, with a significance level of $p \leq 0.05$.

Results

Soil chemical-physical characteristics

Soil chemical-physical characteristics did not show any relevant differences at the beginning of the experiment between I and NI treatments (Table S2). Both soil samples showed high sand content (78–82%) and low clay content (9–10%). The organic matter content was rather low (around 1.37–1.44%) and the pH of both soils was neutral (7.5–7.6). Both soil samples showed a slight level of salinity, as well as low levels of nitrogen and organic matter (Table S2). Similarly, soil chemical-physical characteristics during and at the end of the experimental period did not significantly differ among treatments (Table S3). In IR1 samples, both in August and October, the exchangeable P levels (21.4–20.5 ppm, respectively) were slightly higher compared to all the other treatments (17.88–17.78 ppm, on average, in August and October, respectively), although not significantly. In addition, both nitrogen and organic matter percentages showed a slight decrease in August and October compared to the beginning of the experiment (Table S2–S3). Soil moisture was affected by the irrigation treatment at both sampling times (August and October). Additionally, R3 was always associated to a significantly lower moisture content (Table S4).

Soil enzyme activities

Results on collected soils showed no significant effect of inoculation and inoculation x irrigation interaction on enzyme activity (Table S4). However, a significant effect of irrigation conditions was detected on enzyme activity in samples collected during both sampling times *i.e.*, bulk soils collected in August and in October (Table S4). Particularly, in August, a differential activity of cellobiohydrolase, β -glucosidase and N-acetyl glucosaminidase was detected (Table S4). For all these enzymes, a significant difference was found comparing R1 and R2 to R3, with the R3 samples showing a lower enzymatic activity compared to the others. The same

analysis performed on bulk soils collected in October highlighted that most of the enzymes (*i.e.*, xylosidase, cellobiohydrolase, β -glucosidase, N-acetyl glucosaminidase and acid phosphatase) showed a variation in their activity and, as observed in the first sampling time, the enzymatic activity was significantly lower in R3 (Table S4). Sulfatase did not show any variation in its activity in both times. A higher phosphatase activity was obtained from the samples collected in October compared to those collected in August, whereby the increase was particularly evident for the R1 and R2 irrigation treatments. As stated before, the inoculation showed no impact on soil enzyme activities in the two sampling months. However, although not significant, an increased β -glucosidase activity was observed in I samples when compared to NI ones during both sampling points (Table S4).

Ecophysiological measurements, plant development and production

Stomatal conductance was significantly affected by irrigation, with plants under R1 and R2 showing g_s values that were significantly higher compared to R3

ones. However, no significant difference was observed between R1 and R2 themselves (Table S5 and Fig. 1). Conversely, interaction between inoculation and irrigation did not significantly affect stomatal conductance (Table S5). Concerning the agronomic parameters, inoculation, irrigation and the interaction between these two factors did not result in significant differences in plant height, which ranged between 63.2 ± 8.8 and 73.2 ± 6.2 cm. By contrast, fruit length, fruit width, fruit dry matter, and fruit fresh weight *per plant* were significantly affected by the irrigation treatment (Fig. 2a–d and Tables S6–7). Under R3, these parameters were significantly reduced. Additionally, also the total estimated yield (Fig. 2e) significantly decreased in R3 condition. On the other hand, fruit thickness and fruit number *per plant* were not significantly different among treatments (Table S6–7).

AM fungal colonization in pepper roots

In pepper roots, AM fungal colonization was observed independently from the inoculation (Fig. S3). On the other hand, the irrigation treatment significantly affected M% (intensity of the mycorrhizal colonization

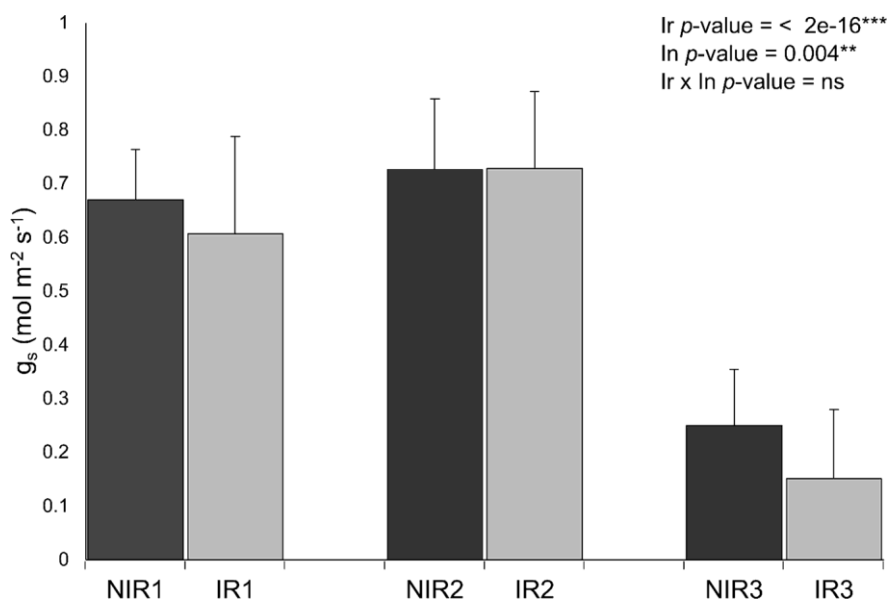


Fig. 1 Stomatal conductance (g_s , mol m⁻² s⁻¹) of pepper leaves. All results are reported as mean $\pm$ standard deviation. Analysis of variance on the single variables is reported in Table S5. Dark grey bars represent not-inoculated samples (NI) and grey bars inoculated ones (I), each subjected to three irrigation treatments (R1 well-watered condition,

R2 reduced irrigation, and R3 rain-fed condition, respectively). ns, *,

, and *: non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Ir: irrigation treatment, In: inoculation, Ir x In: interaction between irrigation treatment and inoculation

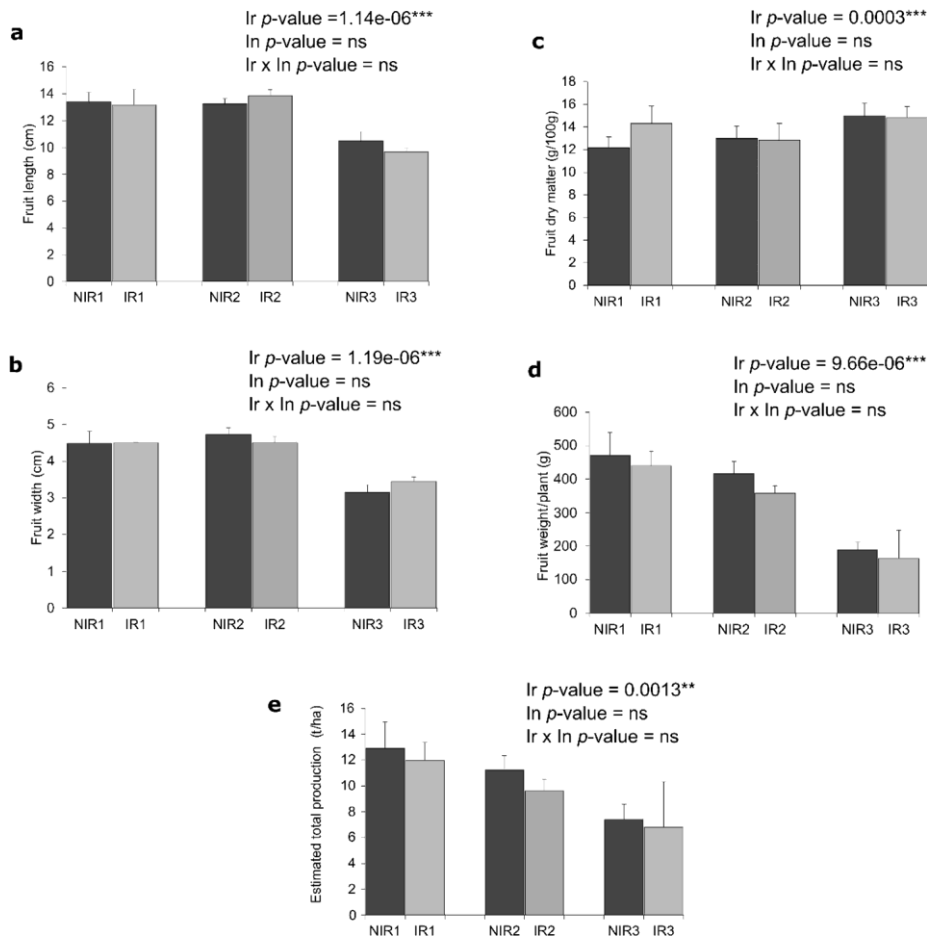


Fig. 2 Agronomic parameters and pepper production. Pepper fruit length (a), fruit width (b), fruit dry matter (g/100 g) (c), fruit weight/plant (g) (d), estimated total yield (t/ha) (e). All results are reported as mean $\pm$ standard deviation. Analysis of variance on the single variables is reported in Tables S6-S7. Dark grey bars represent not-inoculated samples (NI)

and grey bars inoculated ones (I), each subjected to three irrigation treatments (R1 well-watered condition, R2 reduced irrigation, and R3 rain-fed condition, respectively). ns, *, **, and ***: non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Ir: irrigation treatment, In: inoculation, Ir x In: interaction between irrigation treatment and inoculation

in the root system) and A% (arbuscule abundance in the root system), with A% also affected by the irrigation x inoculation interaction (Table S8). The mycorrhizal assessment revealed that both treatments IR3 and NIR3 showed a lower M% and a decreased A% compared to the other treatments (IR1, IR2, NIR1, and NIR2), suggesting a negative impact of the water deficit on the root colonization by the AMF. Specifically, M% was significantly higher in the IR2 treatment than in the NIR3 and IR3 treatments (p -value 0.007), whereas A% was significantly higher in the IR2 treatment compared to all other treatments (p -value 0.001). No significant differences were observed among

treatments regarding F% (frequency of mycorrhiza in the root system), although F% was higher in the IR2 treatment compared to the other treatments (Table S8).

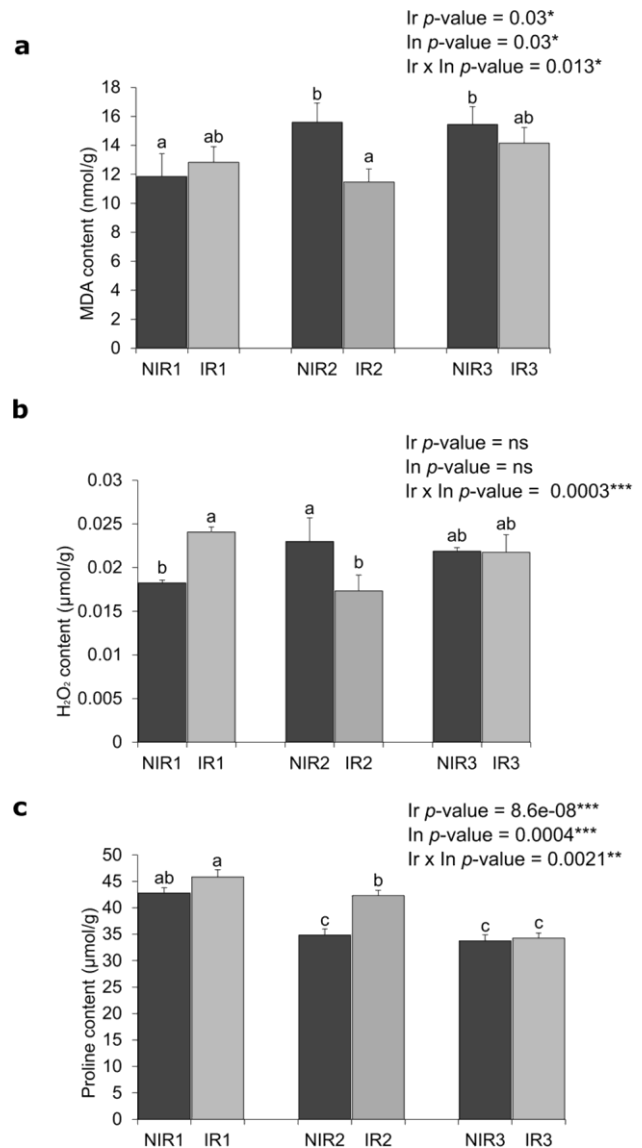
Analysis on biochemical markers and phytohormones in leaves

The results highlighted a significant difference among samples in H_2O_2 considering irrigation x inoculation interaction (Fig. 3, Table S9). Additionally, MDA content and proline were significantly affected by the irrigation and inoculation factors, as well as by the irrigation x inoculation interaction (Table S9). Specifically,

the MDA content was significantly lower in IR2 and NIR1 compared to NIR2 and NIR3 treatments (Fig. 3a), while H_2O_2 content was significantly lower in IR2 and NIR1 treatments compared to the others (Fig. 3b). The highest level of proline content was detected in IR1 and NIR1 (Fig. 3c). However, proline content was also significantly higher in IR2 compared to IR3, NIR2 and NIR3 samples (Fig. 3c). It was possible to observe an inverse trend in proline content with increasing water stress, *i.e.*, samples subjected to R1 showed a higher proline level compared to samples under R2 and R3, both in inoculated and not-inoculated plants. Analysis of plant phytohormones, with a

putative role in tolerance and defense against stressful factors, showed that IAA (indole-3-acetic acid), OPDA (12-oxo-phytodienoic acid) and jasmonic acid (JA) concentrations were significantly affected by inoculation x irrigation interaction (Fig. 4), while abscisic acid (ABA) and salicylic acid (SA) did not show any significant differences considering all the variance factors (Table S10). Considering IAA, a significant difference was found between NIR1 and NIR2 treatments (Fig. 4a). The OPDA concentration was significantly higher in IR2 compared to IR1 and NIR3 (Fig. 4b), while JA content was significantly higher in IR1 compared to all the other treatments (Fig. 4c).

Fig. 3 Biochemical markers (hydrogen peroxide H_2O_2 , malondialdehyde MDA, proline) content in leaves of the different treatments. MDA content (nmol/g) (a), H_2O_2 content ($\mu\text{mol/g } H_2O_2$) (b) and proline content ($\mu\text{mol/g}$) (c) in pepper leaves. All results are reported as mean $\pm$ standard deviation. Different letters represent significant differences according to Tukey HSD test ($p < 0.05$), considering the inoculation x irrigation interaction. Analysis of variance on the single variables is reported in Table S9. Dark grey bars represent not-inoculated samples (NI) and grey bars inoculated ones (I), each subjected to three irrigation treatments (R1 well-watered condition, R2 reduced irrigation, and R3 rain-fed condition, respectively). ns, *, **, and ***: non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Ir: irrigation treatment, In: inoculation, Ir x In: interaction between irrigation treatment and inoculation

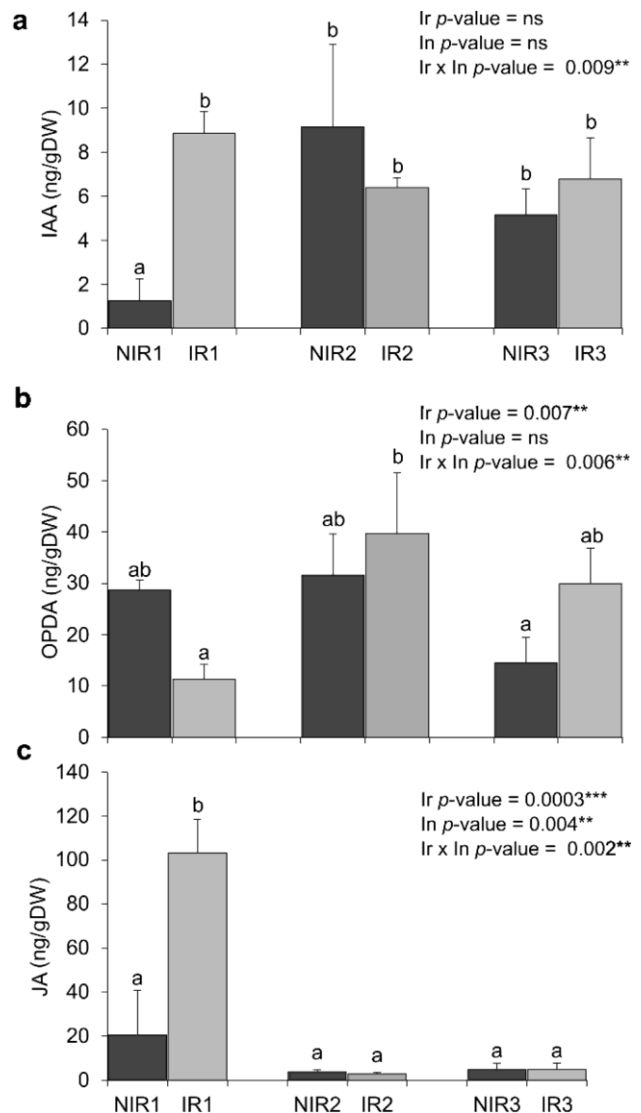


Analysis of antioxidant compounds and micronutrients in fruits

In fruits, all the considered antioxidant compounds were significantly affected by the irrigation x inoculation interaction (Fig. 5, Table S11). In the inoculated condition, anthocyanin content increased in IR2 and IR3 treatments compared to IR1 one (Fig. 5a). On the other hand, under the not-inoculated condition, fruits from NIR3 showed the highest content, followed by NIR1, with NIR2 having the least. Concerning ascorbic acid content (Fig. 5b), NIR3 showed a significantly higher content compared to all the other treatments. Flavonoid content (Fig. 5c) showed a trend considering

the irrigation and the inoculation. Indeed, in the inoculated condition, samples subjected to R1 treatment showed a lower flavonoid content, compared to both R2 and R3 treatments. In fruits of the not-inoculated plants, the lowest flavonoid content was observed in NIR2, while the highest content was recorded in NIR3 treatment. Phenol content (Fig. 5d) increased progressively from IR1 to IR3, and the same trend was also observed in the not-inoculated samples. Overall, the inoculated samples showed a lower phenol content compared to the not-inoculated ones. The DPPH assay results (Fig. 5e) highlighted that, under identical irrigation treatments, both inoculated and not-inoculated samples showed a similar pattern. Indeed, samples

Fig. 4 Hormonal content in pepper leaves (Indole-3-acetic acid – IAA, 12-oxo-phytodienoic acid – OPDA, jasmonic acid – JA). IAA content (ng/g DW) (a), OPDA content (ng/g DW) (b) and JA content (c). All results are reported as mean $\pm$ standard deviation. Different letters represent significant differences according to Tukey HSD test ($p < 0.05$), considering the inoculation x irrigation interaction. Analysis of variance on the single variables is reported in Table S10. Dark grey bars represent not-inoculated samples (NI) and grey bars inoculated ones (I), each subjected to three irrigation treatments (R1 well-watered condition, R2 reduced irrigation, and R3 rain-fed condition, respectively). ns, *, **, and ***: non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Ir: irrigation treatment, In: inoculation, Ir x In: interaction between irrigation treatment and inoculation



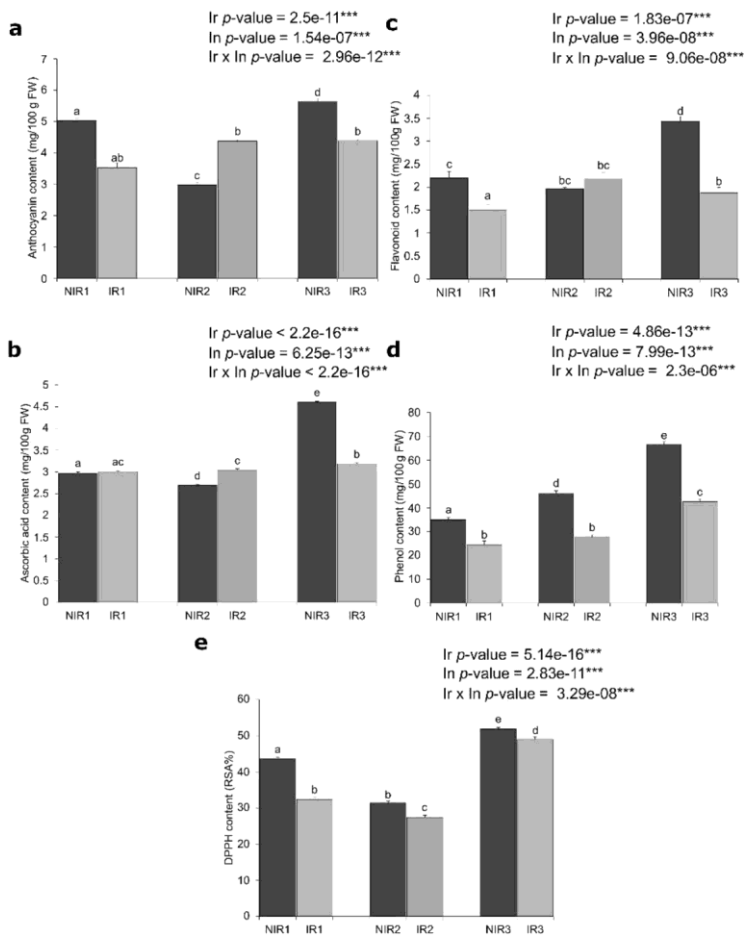


Fig. 5 Antioxidant compounds (anthocyanin, ascorbic acid, flavonoid, phenol, 2,2-difenil-1-picrylhydrazyl – DPPH) in pepper fruits. Anthocyanin content (mg/100 g FW) (a), ascorbic acid content (g/kg FW) (b), flavonoid (mg/100 g FW) (c), phenol content (mg/100 g FW) (d) and DPPH content (RSA%) (e) in fruits. All results are reported as mean $\pm$ standard deviation. Different letters represent significant differences according to Tukey HSD test ($p < 0.05$), considering the inoculation x irrigation interaction. Analysis of variance on the single

variables is reported in Table S11. Dark grey bars represent not-inoculated samples (NI) and grey bars inoculated ones (I), each subjected to three irrigation treatments (R1 well-watered condition, R2 reduced irrigation, and R3 rain-fed condition, respectively). RSA: radical scavenging activity. ns, *, **, and ***: non-significant or significant at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively. Ir: irrigation treatment, In: inoculation, Ir x In: interaction between irrigation treatment and inoculation

subjected to R2 treatment showed a lower DPPH radical scavenging activity compared to samples under R1 and R3 treatments. Moreover, the highest DPPH radical scavenging activity content was found in R3. Overall, a difference between inoculated and not-inoculated condition was identified. A significant lower DPPH radical scavenging activity was found in inoculated samples in all water treatments compared to the not-inoculated ones (Fig. 5e).

Regarding the analysis of fruit micronutrients, the potassium content was influenced by the irrigation

condition, with significantly higher levels observed in samples under R1 compared to those under R3. All the other fruit micronutrients did not significantly differ among treatments (Table S12).

Fungal and bacterial communities in bulk soil and roots over time

The total number of reads obtained from metabarcoding analysis of bulk soil samples were 1,326,631 for fungi and 6,121,663 for bacteria. On the other hand,

in roots, 564,933 and 3,598,961 reads were obtained for fungi and bacteria, respectively. In total, 710 fungal ASVs and 1330 bacterial ASVs were identified and reported in Tables S13 and S14, respectively.

For fungal community, the predominant phyla of bulk soils were represented by phyla Ascomycota, Mortierellomycota, Chytridiomycota and Basidiomycota. In bulk soils collected in August, alpha diversity was affected by the irrigation (p -value 0.043) and by the interaction between the irrigation treatment and the inoculation (p -value 0.029, Fig. 6a). The inoculated samples showed a higher alpha diversity under R1 and R2, while no differences were found comparing the inoculation treatments under R3. Moreover, an increased diversity in terms of taxon abundance from R1 to R3 condition, as well as in I condition compared to NI, was observed (Fig. 6a). Comparative analysis of single factors highlighted that the Ascomycota families Sarcocladiaceae and Onygenales were more prevalent in inoculated samples (Table S15). In bulk soils collected in August, beta diversity revealed significant difference in the fungal communities among different irrigation treatments (p -value 0.026, Fig. S4a) with changes in the relative abundance of the phyla Basidiomycota, Mortierellomycota, Chytridiomycota, and Mucoromycota in relation to the irrigation condition (Fig. 6a). Particularly, the relative abundance of the phylum Chytridiomycota increased from R1 to R3, showing a trend towards dryer conditions (Fig. 6a). Similarly, the relative abundance of Ascomycota was related to the irrigation treatment (Sporormiaceae associated to R1), while relative abundance of Basidiomycota *i.e.*, Piskurozymaceae was associated to R3 (Table S15). Moreover, a difference in relative taxon abundance for Chaetomiaceae and Glomerallaceae (Ascomycota), Calcarisporiellaceae (Mucoromycota), Filobasidiaceae (Basidiomycota) and Chytridiaceae (Chytridiomycota) was observed among specific treatments (Table S15).

Fungal alpha diversity of bulk soil samples collected in October did not reveal any differences between treatments (Fig. 6b), while beta diversity highlighted a significant difference among R1, R2 and R3 (p -value 0.001, Fig. S4b). In bulk soils collected in October, among Ascomycota, Pezizaceae family was relatively more abundant in samples subjected to R1 condition, while Microdochiaceae was relatively more abundant in samples subjected to R2 and

R3 condition (Table S16). On the other hand, among Basidiomycota, Symmetrosporaceae, Holtermanniaceae (family *incertae sedis*) and Chysozymaceae were uniquely associated to samples subjected to R3 condition (Table S16). A different taxon composition among treatments was also identified (Table S16).

The core microbiome of fungal community associated to pepper root samples was represented by phyla Ascomycota, Basidiomycota, and Mortierellomycota (Fig. 6c). Additionally, phylum Mortierellomycota was associated only with NI samples. Alpha diversity showed a significant difference between I and NI condition (p -value 0.033; Fig. 6c). A decreasing trend in the taxon abundance considering the different irrigation treatments was observed (Fig. 6c). Beta diversity revealed a significant difference inside the fungal communities at different irrigation treatments (p -value 0.008; Fig. S4c). In addition, the inoculated samples showed an overall higher taxon abundance compared to the NI ones, and the irrigation treatment showed a selective effect, *i.e.*, taxa progressively decreased from R1 to R3. In particular, NIR3 treatment was characterized by the lowest associated fungal diversity mostly represented by Basidiomycota (Fig. 6c). The families Microdochiaceae of Ascomycota and Filobasidiaceae of Basidiomycota showed a higher taxon abundance in NI samples (Table S17). A trend was observed in relation to the irrigation treatments. Ascomycota as Glomeraceae and Didymosphaeriaceae were less abundant in R3, while Chaetomiaceae and Didymellaceae were more abundant. Similarly, Glomerellaceae were associated to R1. The lowest abundance of Mucormomycota *i.e.*, Mortierellaceae was detected in NIR3 treatment. The different treatments significantly affected taxon distribution, as reported in Table S17.

Concerning bacterial communities, both bulk soils collected in August and October showed diverse abundances of different phyla *i.e.*, Proteobacteria, Bacteroidota, and Actinobacteriota, except for Firmicutes uniquely associated to samples collected in October (Fig. 7a–b). In August, beta diversity highlighted a significant difference between I and NI, while no significant differences were detected in samples collected in October (p -value 0.004, Fig. S5a–b). The NIR1 treatment showed the higher number of taxa compared to the other treatments, mainly due to the abundance of members of the phyla Thermoproteota, Acidobacteriota, Chloroflexota and

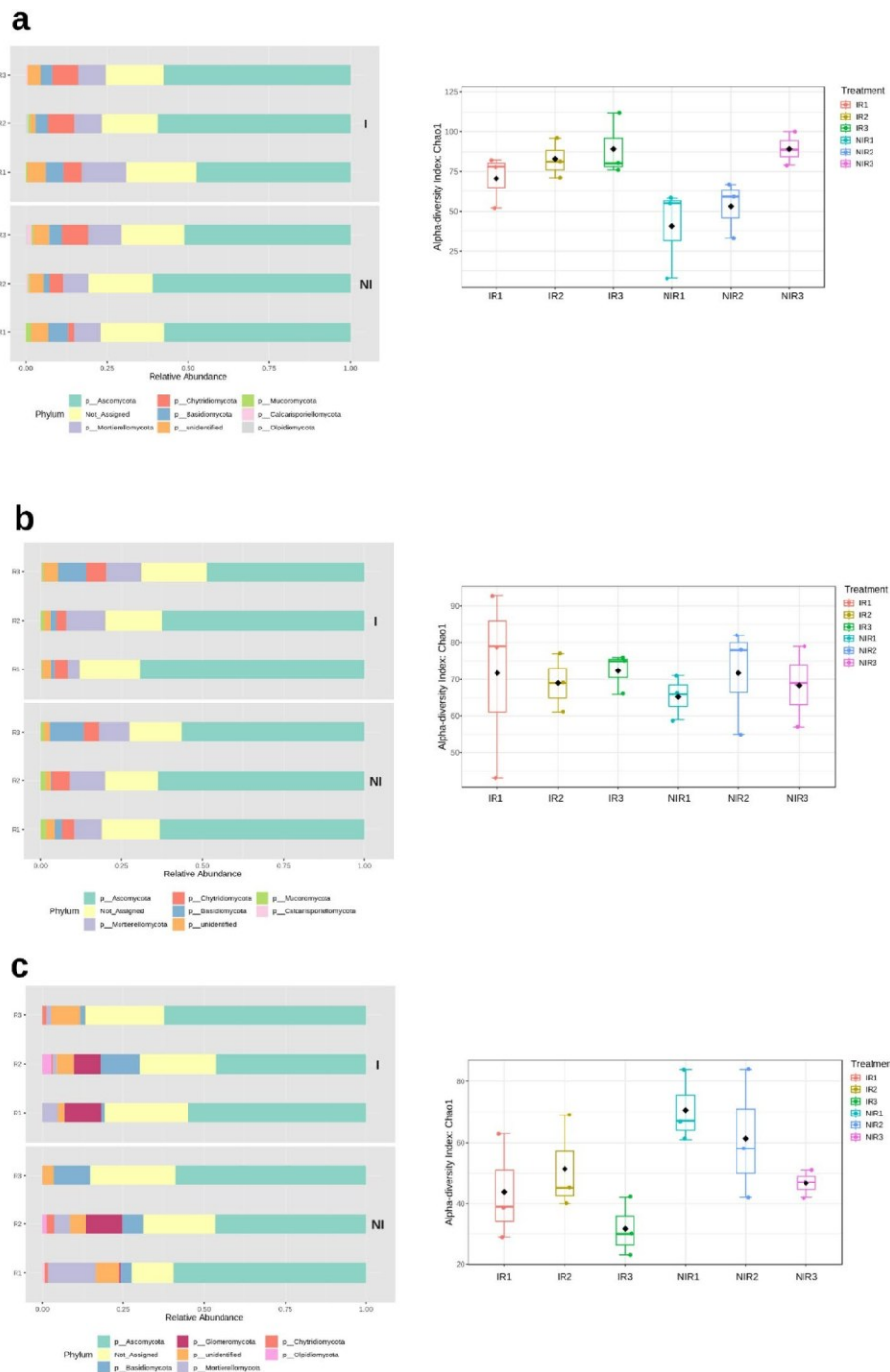


Fig. 6 Assessment of fungal diversity within samples. Taxon bar plot and alpha diversity of bulk soils collected in August (a), bulk soils collected in October (b) and roots collected in October (c). Taxon bar plots were produced at phylum level considering the inoculation treatment (inoculated samples – I and not-inoculated samples – NI, each subjected to three irrigation treatments (R1 well-watered condition, R2 reduced

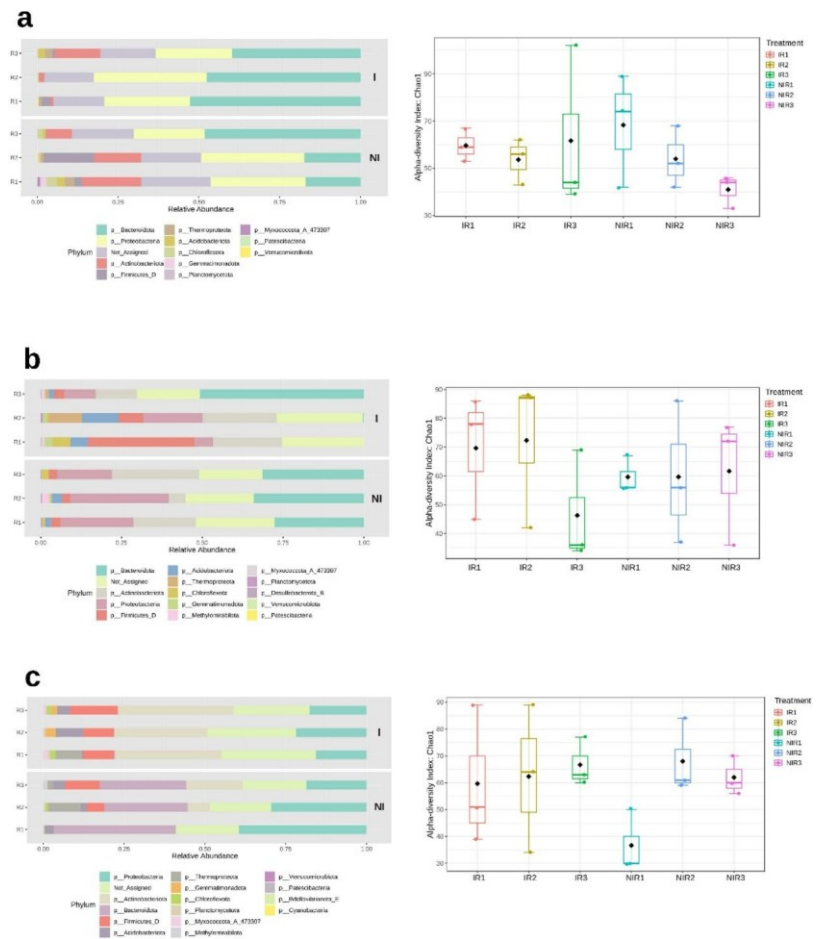
irrigation, and R3 rain-fed condition, respectively). Alpha diversity was performed considering all treatments *i.e.*, IR1–inoculated samples in well-watered condition, IR2–inoculated samples in reduced irrigation, IR3–inoculated samples in rain-fed condition, NIR1–not-inoculated samples in well-watered condition, NIR2–not-inoculated samples in reduced irrigation, NIR3–not-inoculated samples in rain-fed condition

Gemmatimonadota, while in I samples, members of the phylum Bacteroidota were more abundant (Fig. 7a). The Rhizobiaceae family within Proteobacteria was the most abundant one in all I samples (Table S18). Bulk soils collected in October showed a higher abundance of Proteobacteria and Bacteroidota in NI samples and a higher abundance of Firmicutes and Acidobacteriota in I ones. Notably, Bacteroidota was more abundant in IR3 compared to IR1 and IR2 (Fig. 7b). In October, the inoculation significantly changed taxon distribution among treatments (Table S19). Geodermatophilaceae family (Actinobacteria) was more abundant in inoculated samples. On the contrary, Marinilabiliaceae (Bacteroidota) and Lacipirellulaceae (Planctomycetota) were more abundant in NI. Notably, Marinilabiliaceae were also abundant in IR3 treatment. The irrigation treatment had a significant effect on the bacterial communities for both sampling times (Table S18–19). In

August, the highest abundance of Burkholderiaceae, Azospirillaceae and Xanthobacteraceae was observed in R3 (Table S18). In October, a family within Actinobacteria, *i.e.*, Gaiellaceae, was more abundant in R1 and R2, while a family within Proteobacteria *i.e.*, Beijerinckiaceae was associated to R3 condition (Table S19). Moreover, a shift in taxon distribution among treatments was observed in samples collected in October (Table S19).

The core microbiome at phylum level of bacterial communities in roots was represented by Proteobacteria, Actinobacteriota, Firmicutes, and Acidobacteriota. Bacteroidota were more abundant in NI, while Acidobacteriota, Actinobacteriota, Gemmatimonadota, Chloroflexota and Firmicutes were more abundant in I samples (Fig. 7c). Beta diversity showed a significant difference in taxon abundance among treatments (p-value 0.009; Fig. S5c). Both the inoculation and the irrigation resulted in a significant effect

Fig. 7 Assessment of bacterial diversity within samples. Taxon bar plot and alpha diversity of bulk soils collected in August (a), bulk soils collected in October (b) roots collected in October (c). Taxon bar plots were produced at phylum level considering the inoculation treatment (inoculated samples – I and not-inoculated samples – NI, each subjected to three irrigation treatments (R1 well-watered condition, R2 reduced irrigation, and R3 rain-fed condition, respectively). Alpha diversity was performed considering all treatments *i.e.*, IR1–inoculated samples in well-watered condition, IR2–inoculated samples in reduced irrigation, IR3–inoculated samples in rain-fed condition, NIR1–not-inoculated samples in well-watered condition, NIR2–not-inoculated samples in reduced irrigation, NIR3–not-inoculated samples in rain-fed condition



on taxon distribution (Table S20). Solirubrobacterales family (Actinobacteria) was more abundant in R3. Marinilabiliaceae (Bacteroidota), Rhizobiaceae and Cellvibrionaceae (Proteobacteria) were more abundant in NI, while Gaiellaceae family (Actinobacteria) was associated to I samples (Table S20).

Discussion

The impact of the irrigation treatments on pepper eco-physiological and agronomic parameters

Our field trial study provided a comprehensive understanding on the responses of pepper to water deficit and AM fungal inoculation. The study revealed that g_s was not affected by reduced irrigation (R2), while it declined significantly in rain-fed pepper (R3). Results of the agronomic parameters showed that fruit length and width were significantly impacted by the irrigation treatment, as previously observed in pepper (Feng et al. 2019; Khazaei et al. 2020), highlighting that the water treatment is one of the key drivers affecting pepper growth and yield features. Indeed, the reduction in fruit size and weight under rain-fed (R3) compared to well-watered conditions (R1) or reduced irrigation (R2) underscores the reported responses of pepper plants to limited water availability (Ali et al. 2022; Khazaei et al. 2020). It has been reported that bell pepper cv Aristotle irrigated at 67% ETc had similar fruit yield and quality compared to plants at 100% ETc (Kabir et al. 2021). Interestingly, our results showed no significant differences in pepper production between R1 and R2 condition, thus suggesting a potential application of a reduced water supply to cultivate pepper plants.

The role of the AM fungal inoculum on plant responses to irrigation treatments

In addition to exploring the effects of water treatments in pepper physiology, the main aim of this study was to investigate the interactions between AMF and the colonized pepper plants in response to diverse levels of water availability, including the structure and dynamics of root and bulk soil-associated microbiomes. In our experimental field, not-inoculated plants also showed root colonization by native AMF. This is in line with a recent comparative analysis on barley and sorghum

AMF-inoculated plants showed no significant differences in fungal colonization by native soil AM fungal communities and commercial inoculum (Frew 2021). However, a combined effect of the native and externally applied AM fungal inoculum was detected for some measured plant parameters, probably due to a boost by the commercial inoculum. It is worth noting that although AM fungal inoculation has been reported to have a positive impact on plant growth and productivity in diverse species, including several crops, the effect of field inoculations with AMF is highly unpredictable and variable (Lutz et al. 2023). The results of the assessment of AM fungal colonization in root samples revealed that the IR2 treatment exhibited the highest colonization levels. This finding is in keeping with the agronomic and ecophysiological results, suggesting that the reduced irrigation applied in this study (*i.e.*, 75% of the well-watered treatments—R2) may be considered optimal also for AM fungal colonization. Conversely, plants subjected to the R3 treatment showed lower level of AM fungal colonization, with a reduced presence of arbuscules.

Despite the negative effect that water deficit may have on AM fungal colonization (Wu and Zou 2017), results demonstrated that AM fungal inoculation improved several plant parameters. Notably, flavonoid content in pepper leaves was higher in inoculated (I) condition, indicating a potential enhancement in the plant stress response and production of secondary metabolites, such as flavonoids, due to the symbiotic relationship with AMF, as previously reported (Castellanos-Morales et al. 2010; Pal et al. 2024). The analysis of stress markers MDA, H_2O_2 , and proline showed variations based on the interaction between irrigation and inoculation. Based on our proline data, we can hypothesize that the well-watered treatment (100% of ETc—R1) may result in excess water retention in the considered soil. Consequently, plants might perceive a mild excess of water in the rhizosphere and activate responses to water stress, leading to proline accumulation in R1 leaves (particularly in IR1 and NIR1 treated plants). This is coupled with slightly lower g_s rates compared to R2 treated plants, similarly to previous observations in green sweet pepper plants subjected to both drought and flooding stresses (Masoumi et al. 2021). These findings, in agreement with the susceptibility of pepper to both water deficit and water excess (Masoumi et al. 2021), suggest that precise estimation of ETc for a specific crop is a critical issue for

effective irrigation management in soils with diverse water storage capacities. This highlights the necessity of defining tailored crop coefficients (K_c) for particular climatic areas (Miranda et al. 2006). Notably, the higher proline content in IR2 treatment compared to NIR2 may suggest the ability of AMF in improving plant stress response, as previously observed (Zheng et al. 2020; Zou et al. 2021). In this line, the significant high content in IR2 of the phytohormone OPDA, a precursor of JA, known to have a key role in triggering signaling pathways that regulate JA-responsive genes (Liu and Park 2021), may also suggest a role of AMF in the observed OPDA increase. Additionally, IAA content in IR1 treatments can also be linked to the presence of AMF. It has been previously reported that inoculation with *F. mosseae* can induce the production of OPDA and IAA in tomato plants and in trifoliate orange, respectively (Liu et al. 2018; López-Ráez et al. 2010).

Fruit antioxidant compounds were affected both by irrigation and inoculation. Plants grown in the R3 treatment showed a higher antioxidant compounds abundance compared to R1 and R2. Moreover, considering the inoculation, a common trend was observed: NI samples showed a higher content of antioxidant compounds, compared to I samples. Despite several studies have tried to correlate AM fungal inoculation and antioxidant content in fruits (Abdelhalim et al. 2022; Grozić et al. 2021), there is not a definitive answer, because the presence of antioxidants may depend on multiple parameters (e.g., exposure to extreme temperatures, drought, salinity) (Baslam and Goicoechea 2012; Mishra et al. 2023; Torres et al. 2018). In our case, it is worth noting that anthocyanin and ascorbic acid increased in IR2 treatment compared to NIR2, suggesting a pivotal role of the irrigation treatment combined with AM fungal inoculation in pepper plants. This relation is inverted for phenol and DPPH content: IR2 treatment showed a lower content compared to NIR2. The DPPH radical scavenging activity indicates the antioxidant plant capacity and is activated under abiotic stresses, as water deficit conditions (Abdalla et al. 2019), therefore its reduction under AM fungal inoculation may suggest a reduced stress level in inoculated samples. A similar role has been proposed for phenol (Rosa-Martínez et al. 2023), suggesting that, in our conditions, pepper plants in IR2 treatment produced more anthocyanin and ascorbic acid and less DPPH and

phenol, compared to NIR2, shaping their response in relation to the different conditions. Flavonoid content highlighted a similar trend among the irrigation treatments and between the two inoculation treatments. In this case, there were no significant differences between IR2 and NIR2, but it is noteworthy that: i) IR2 is associated to the highest flavonoid abundance among the inoculated samples, and ii) NIR2 is associated to the lowest flavonoid abundance among the not-inoculated ones. These data suggest that IR2 may be the optimal condition to boost flavonoid content in pepper fruits and the associated antioxidant potential (Hernández-Pérez et al. 2020). Concerning pepper fruit nutrients, the only nutrient showing significant variation was potassium, with a decreasing content from well-watered (R1) to rain-fed conditions (R3). This could indicate that water availability plays a crucial role in nutrient uptake, especially for potassium. It has been reported that the potassium content can be related to water availability in pepper plants, showing a significant decrease under water deficit conditions (Kabir et al. 2021). Overall, our results suggested that antioxidant compound production may vary depending on multiple factors, and that AM fungal inoculation might trigger specific compound production, as recently documented (Shalaby and El-Sayed Ramadan 2024). Further studies are needed to understand how application of AM fungal inoculation may shape pepper fruit quality.

The impact of the irrigation and the AM fungal inoculum on the soil and root-associated microbial communities

Metabarcoding analysis revealed that Chytridiomycota phylum was more abundant in bulk soils collected in August mainly in R3 treatment and, in agreement with this result, this phylum is known to include members showing the ability to cope with water deficit condition (Dacal et al. 2022). Moreover, R3 bulk soils collected in August revealed an enrichment in Burkholderiaceae, Azospirillaceae and Xanthobacteraceae. Burkholderiaceae family was also detected in bulk soil collected in October in NIR2 and NIR3, as well as Azospirillaceae in R3 samples. These data are in line with previous studies (Jang et al. 2020; Maestro-Gaitán et al. 2023; Munoz-Ucros et al. 2022; Yasuda et al. 2022). Burkholderiaceae and Xanthobacteraceae are reported to increase under water deficit condition, resulting in

improving plant in carbon cycling and nitrogen fixation and plant response to phytopathogen (Jang et al. 2020; Maestro-Gaitán et al. 2023; Munoz-Ucros et al. 2022). Conversely, Azospirillaceae family has been reported as a plant growth promoting bacteria (PGPB) able to improve nitrogen mineralization under water deficit conditions (Yasuda et al. 2022). A high abundance of Myxococcaceae was detected in IR1 and IR3, both in bulk soils and in root samples collected in October. Myxococcaceae are known to be able to survive in adverse environments, producing antibiotics and important secondary metabolites, such as carotenoids, able to improve plant tolerance against several phytopathogens (Zhou et al. 2021). The order Myxococcales seems to be associated to AM fungal hyphae (Emmett et al. 2021), and our results showed that this order was more abundant in inoculated samples. Notably, pathogenic fungal taxon Didymellaceae (Chen et al. 2017) was detected in roots of R3 and IR3 plants. The concurrent detection of Myxococcales and fungal pathogen taxa, specifically Didymellaceae, in the roots of inoculated plants, may suggest that AMF can recruit biological control agents. Interestingly, according to Lutz et al. (2023), the presence of plant pathogens may be used to assess AMF potential in restoring plant health, as it has been documented that AM fungal inoculation has a relevant impact on pathogen-infected soil, enhancing the plant response to pathogens and overall performance compared to not-inoculated soils. In our experiment, in addition, it is worth noting that bulk soil from inoculated plants showed a high abundance of Geodermatophilaceae, a bacterial family that has been identified as a component of the mycorrhizosphere and hyphosphere microbiome of AMF (Zhang et al. 2022b).

Results on soil enzyme activity suggested that irrigation treatments are the primary determinant of the enzyme activity, as previously reported in other field experiments with maize (Muhammad et al. 2022) and alfalfa (Deng et al. 2022). The consistently lower enzymatic activity observed in R3 across both sampling times for most of the enzymes studied is of particular note. This trend suggested that the R3 irrigation treatment may not be favorable in increasing microbial activity and enzyme production, as instead observed in R1 and R2. The high fungal alpha diversity in R1 and R2 could be associated to an enhanced enzyme activity, putatively linked to organic matter decomposition and nutrient cycling. The differential activity of cellobiohydrolase, β -glucosidase, and N-acetyl glucosaminidase

in August, with the addition of xylosidase, cellulase, and acid phosphatase in October, suggested that soil enzyme activities are also subjected to fluctuations influenced by a range of factors, including soil moisture levels and climatic changes. Overall, soil enzymatic activity was significantly affected by irrigation treatments, while AM fungal inoculation has no relevant effect, with the exception of β -glucosidase activity. Hence, we cannot exclude that the strong effect of water regime may have potentially masked the effects mediated by AM fungal inoculation. It is worth noting that not-inoculated treatments also showed the presence of native AM fungal communities, resulting in similar soil enzymatic activity in comparison with inoculated ones. Nevertheless, inoculation predominantly affected β -glucosidase activity. This outcome could be explained by the presence of bacteria of the Rhizobiaceae family being more abundant in inoculated treatments than in not-inoculated and known for a high production of β -glucosidase (Duan et al. 2022). Lastly, an increased phosphatase activity was observed in October compared to August, especially under the R1 and R2 treatments. Members of Firmicutes, often associated with high phosphatase activity (Cui et al. 2019; Li et al. 2020), and exclusively found in October samples, may be indicative of the enhanced enzymatic activity recorded in this period.

Looking at the roots, our results showed that rain-fed condition (R3) was associated with an enrichment in plant-beneficial taxa such as Firmicutes and Gemmatimonadota, as already reported in other crops (Faghini et al. 2023; Santos-Medellín et al. 2021). In addition, Actinobacteria phylum was abundant in inoculated roots under R3 treatment, underlining a hypothetical role of AMF in recruiting beneficial taxa as PGPB under stressed conditions, as previously reported (Garbaye 1991; Sbrana et al. 2022). Concerning fungal communities, mycorrhizal assessment in roots showed that IR2 was the most AMF-colonized treatment. Focusing on AM fungal communities, metabarcoding analysis showed that the Glomeraceae family (Spatofora et al. 2016) was found to be abundant in inoculated roots, although its presence was significantly affected by the irrigation treatment, with the lowest abundance detected in R3, in agreement with previous results reporting that drought negatively affects AM fungal colonization (Wu and Zou 2017). On the other hand, samples subjected to R1 and R2 irrigation treatments showed no statistical differences in Glomeraceae abundance, highlighting that

R2 may be similar to R1 (well-watered), especially from a sustainability point of view where saving water is considered as a key approach.

In conclusion, our results contribute to enhance the understanding of pepper plant traits affected by AM fungal inoculation and water deficit conditions. Our findings confirmed a positive impact of the application of an AM fungal inoculum on pepper plants and suggested that, at least for the considered pepper genotype, reduced irrigation (75% of ETc) could not cause stress conditions, ensuring significant water savings. Additionally, our study sheds light on the intricate interactions among bacteria, fungi (AMF and non-AMF), and pepper roots under water limited conditions. Overall, our study underlines the interconnected nature of these factors, emphasizing the need to consider their interdependence rather than evaluating them alone.

Acknowledgements The authors thank Francesca Brescia and Yulia Kiryakova for the help in DNA extraction and eco-physiological measurements, respectively, in addition to Beatrix Schnabel from the Department of Soil Ecology (UFZ) for the support in the sequencing analysis.

Author contribution Conceptualization: RB, CM, VM; Methodology: RB, TR, FS, WC; Investigation: ACa, TR, FS, VM, EC, EZ, RM, GG, LG, ACo, CM, GP, MGG, VA, VF; Formal analysis: FS, TR, LG; Writing—Original Draft: ACa, FS, VM, MGG, EZ; Writing—Review & Editing: ACa, TR, FS, ACo, WC, MC, VF, RB; Supervision: RB.

Funding Open access funding provided by Consiglio Nazionale Delle Ricerche (CNR) within the CRUI-CARE Agreement. The work was partially funded by the PRIMA RESCHEDULE project (Italian MUR DD 1293/2021 and German BMBF 01DH21019) and by CNR project FOE-2021 DBA.AD005.225.

Data availability Reads from metabarcoding of pepper soil and root samples were submitted to NCBI SRA under BioProject PRJNA1024920.

Declarations

Competing interests The authors declare that there are no conflicts of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated

otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abdalla MUE, Taher M, Sanad MI, Tadros LK (2019) Chemical properties, phenolic profiles and antioxidant activities of pepper fruits. *J Agric Chem Biotechnol* 10:133–140. <https://doi.org/10.21608/jacb.2019.53475>
- Abdelhalim TS, Tia NAJ, Sir Elkhatim KA, Othman MH, Joergensen RG, Almaiman SA, Hassan AB (2022) Exploring the potential of arbuscular mycorrhizal fungi (AMF) for improving health-promoting phytochemicals in sorghum. *Rhizosphere* 24:100596. <https://doi.org/10.1016/j.rhisph.2022.100596>
- Abdelkhalik A, Abd El-Mageed TA, Mohamed IAA, Semida WM, Al-Elwany OAAI, Ibrahim IM, Hemida KA, El-Saadony MT, Abu Qamar SF, El-Tarabily KA, Gyushi MAH (2023) Soil application of effective microorganisms and nitrogen alleviates salt stress in hot pepper (*Capsicum annum* L.) plants. *Front Plant Sci* 13. <https://www.frontiersin.org/articles/10.3389/fpls.2022.1079260>
- Ahluwalia O, Singh PC, Bhatia R (2021) A review on drought stress in plants: implications, mitigation and the role of plant growth promoting rhizobacteria. *Resour Environ Sustain* 5:100032. <https://doi.org/10.1016/j.resenv.2021.100032>
- Ali S, Tyagi A, Park S, Mir RA, Mushtaq M, Bhat B, Mahmoudi H, Bae H (2022) Deciphering the plant microbiome to improve drought tolerance: mechanisms and perspectives. *Environ Exp Bot* 201:104933. <https://doi.org/10.1016/j.envexpbot.2022.104933>
- Allen R, Pereira L, Raes D, Smith M (1998) Crop evapotranspiration guidelines for computing crop requirements. FAO Irrig. Drain. Report modeling and application. *J Hydrol* 285:19–40
- Alvino A, Centritto M, De Lorenzi F (1994) Photosynthesis response of sunlit and shade pepper (*Capsicum annum*) leaves at different positions in the canopy under two water regimes. *Funct Plant Biol* 21:377–391. <https://doi.org/10.1071/PP9940377>
- Augé RM, Toler HD, Saxton AM (2015) Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza* 25:13–24. <https://doi.org/10.1007/s00572-014-0585-4>
- Ávila-Dávila L, Molina-Martínez JM, Bautista-Capetillo C, Soler-Méndez M, Robles Rovelo CO, Júnez-Ferreira HE, González-Trinidad J (2021) Estimation of the evapotranspiration and crop coefficients of bell pepper using a removable weighing lysimeter: a case study in the southeast of Spain. *Sustainability* 13:747. <https://doi.org/10.3390/su13020747>

- Baslam M, Goicoechea N (2012) Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. *Mycorrhiza* 22:347–359. <https://doi.org/10.1007/s00572-011-0408-9>
- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* 39:205–207. <https://doi.org/10.1007/BF00018060>
- Berendsen RL, Pieterse CM, Bakker PA (2012) The rhizosphere microbiome and plant health. *Trends Plant Sci* 17:478–486. <https://doi.org/10.1016/j.tplants.2012.04.001>
- Bhattacharyya A, Pablo CH, Mavrodi OV, Weller DM, Thomashow LS, Mavrodi DV (2021) Rhizosphere plant-microbe interactions under water stress. *Adv Appl Microbiol* 115:65–113. <https://doi.org/10.1016/bs.aambs.2021.03.001>
- Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardson CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciulek T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Loftfield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIVER LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Nothias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Priesse E, Rasmussen LB, Rivers A, Robeson MS 2nd, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37:852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Brunetti C, Saleem AR, Della Rocca G, Emiliani G, De Carlo A, Balestrini R, Khalid A, Mahmood T, Centritto M (2021) Effects of plant growth-promoting rhizobacteria strains producing ACC deaminase on photosynthesis, isoprene emission, ethylene formation and growth of *Mucuna pruriens* (L.) DC. in response to water deficit. *J Biotechnol* 331:53–62. <https://doi.org/10.1016/j.jbiotec.2021.03.008>
- Caddell D, Louie K, Bowen B, Sievert J, Hollingsworth J, Dahlberg J, Purdom E, Northen T, Coleman-Derr D (2023) Drought shifts sorghum root metabolite and microbiome profiles and enriches the stress response factor piceolic acid. *Phytophormones J* 4:449–463. <https://doi.org/10.1094/PBIOMES-02-23-0011-R>
- Castellanos-Morales V, Villegas J, Wendelin S, Vierheilig H, Eder R, Cárdenas-Navarro R (2010) Root colonisation by the arbuscular mycorrhizal fungus *Glomus intraradices* alters the quality of strawberry fruits (*Fragaria × ananassa* Duch.) at different nitrogen levels. *J Sci Food Agric* 90:1774–1782. <https://doi.org/10.1002/jsfa.3998>
- Chen Q, Hou LW, Duan WJ, Crous PW, Cai L (2017) Didymellaceae revisited. *Stud Mycol* 87:105–159. <https://doi.org/10.1016/j.simyco.2017.06.002>
- Chitarra W, Pagliarini C, Maserti B, Lumini E, Siciliano I, Cascone P, Schubert A, Gambino G, Balestrini R, Guerrieri E (2016) Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress. *Plant Physiol* 171:1009–1023. <https://doi.org/10.1104/pp.16.00307>
- Chong J, Liu P, Zhou G, Xia J (2020) Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc* 15:3. <https://doi.org/10.1038/s41596-019-0264-1>
- Christou A, Manganaris GA, Papadopoulos I, Fotopoulos V (2013) Hydrogen sulfide induces systemic tolerance to salinity and non-ionic osmotic stress in strawberry plants through modification of reactive species biosynthesis and transcriptional regulation of multiple defense pathways. *J Exp Bot* 64:1953–1966. <https://doi.org/10.1093/jxb/ert055>
- Cui Y, Fang L, Guo X, Wang X, Wang Y, Zhang Y, Zhang (2019) Responses of soil bacterial communities, enzyme activities, and nutrients to agricultural-to-natural ecosystem conversion in the Loess Plateau. *China J Soils Sediments* 19:1427–1440. <https://doi.org/10.1007/s11368-018-2110-4>
- Dacal M, García-Palacios P, Asensio S, Wang J, Singh BK, Maestre FT (2022) Climate change legacies contrastingly affect the resistance and resilience of soil microbial communities and multifunctionality to extreme drought. *Funct Ecol* 36:908–920. <https://doi.org/10.1111/1365-2435.14000>
- De Ollas C, González-Guzmán M, Pitarch Z, Matus JT, Candela H, Rambla JL, Granell A, Gómez-Cadenas A, Arbona V (2021) Identification of ABA-mediated genetic and metabolic responses to soil flooding in tomato (*Solanum lycopersicum* L. Mill). *Front Plant Sci* 12:613059. <https://doi.org/10.3389/fpls.2021.613059>
- de Vries FT, Griffiths RI, Knight CG, Nicolitch O, Williams A (2020) Harnessing rhizosphere microbiomes for drought-resilient crop production. *Science* 368:270–274. <https://doi.org/10.1126/science.aaz5192>
- Dehghan G, Khoshkam Z (2012) Tin (II)–quercetin complex: synthesis, spectral characterisation and antioxidant activity. *Food Chem* 131:422–426. <https://doi.org/10.1016/j.foodchem.2011.08.074>
- Delfine S, Loreto F, Alvino A (2001) Drought-stress effects on physiology, growth and biomass production of rainfed and irrigated bell pepper plants in the Mediterranean region. *J Am Soc Hortic Sci* 126:297–304. <https://doi.org/10.21273/JASHS.126.3.297>
- Deng Q, Wu Y, Zhao X, Qiu C, Xia S, Feng Y, Liu H (2022) Influence of different irrigation methods on the alfalfa rhizosphere soil fungal communities in an arid region. *PLoS ONE* 17. <https://doi.org/10.1371/journal.pone.0268175>
- Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J (2017) MicrobiomeAnalyst: a web-based tool for

- comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* 45:180–188. <https://doi.org/10.1093/nar/gkx295>
- Duan C, Mei Y, Wang Q, Wang Y, Li Q, Hong M, Hu S, Li S, Fang L (2022) *Rhizobium* inoculation enhances the resistance of alfalfa and microbial characteristics in copper-contaminated soil. *Front Microbiol* 12:781831. <https://doi.org/10.3389/fmicb.2021.781831>
- Emmett BD, Lévesque-Tremblay V, Harrison MJ (2021) Conserved and reproducible bacterial communities associate with extraradical hyphae of arbuscular mycorrhizal fungi. *ISME J* 15:2276–2288. <https://doi.org/10.1038/s41396-021-00920-2>
- Fadiji AE, Santoyo G, Yadav AN, Babalola OO (2022) Efforts towards overcoming drought stress in crops: revisiting the mechanisms employed by plant growth-promoting bacteria. *Front Microbiol* 13. <https://www.frontiersin.org/articles/10.3389/fmicb.2022.962427>
- Faghihinia M, Jansa J, Halverson LJ, Staddon PL (2023) Hyphosphere microbiome of arbuscular mycorrhizal fungi: a realm of unknowns. *Biol Fertil Soils* 59:17–34. <https://doi.org/10.1007/s00374-022-01683-4>
- FAOSTAT (2021) Food and Agriculture Organization of the United Nations. Accessed 01.09.2023. FAOSTAT. <https://www.fao.org/statistics/en>
- Feng X-H, Zhang H-X, Ali M, Gai W-X, Cheng G-X, Yu Q-H, Yang S-B, Li X-X, Gong Z-H (2019) A small heat shock protein *CaHsp25.9* positively regulates heat, salt, and drought stress tolerance in pepper (*Capsicum annuum* L.). *Plant Physiol Biochem* 142:151–162. <https://doi.org/10.1016/j.plaphy.2019.07.001>
- Food insecurity (2022) *Nat Clim Chang* 12:963. <https://doi.org/10.1038/s41558-022-01530-2>
- Frew A (2021) Contrasting effects of commercial and native arbuscular mycorrhizal fungal inoculants on plant biomass allocation, nutrients, and phenolics. *Plants People Planet* 3:536–540. <https://doi.org/10.1002/ppp3.10128>
- Garbaye J (1991) Biological interactions in the mycorrhizosphere. *Experientia* 47:370–375. <https://doi.org/10.1007/BF01972079>
- German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD (2011) Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biol Biochem* 43:1387–1397. <https://doi.org/10.1016/j.soilbio.2011.03.017>
- Giusti MM, Wrolstad E (2001) Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Curr Protoc* 00:F1.2.1-F1.2.13. <https://doi.org/10.1002/047142913.faf0102s00>
- Grozić K, Pasković I, Radić T, Lukić M, Žurga P, Palčić I, Urlić B, Brkljača M, Ban D, Kaliterna J, Klanjac J, Major N, Matotán Z, Goreta Ban S (2021) Processing tomato response to arbuscular mycorrhizal fungi application under conventional production practice. *J Cent Eur Agric* 22:72–85. <https://doi.org/10.5513/JCEA01/22.1.2856>
- Güneş H, Demir S, Erdinc C, Furan A (2023) Effects of arbuscular mycorrhizal fungi (AMF) and biochar on the growth of pepper (*Capsicum annuum* L.) under salt stress. *Gesunde Pflanz* 75:1–13. <https://doi.org/10.1007/s10343-023-00897-2>
- Haworth M, Marino G, Loreto F, Centritto M (2021) Integrating stomatal physiology and morphology: evolution of stomatal control and development of future crops. *Oecologia* 197:867–883. <https://doi.org/10.1007/s00442-021-04857-3>
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Int Physiol Biochem Biophys* 125:189–98. [https://doi.org/10.1016/0003-9861\(68\)90654-1](https://doi.org/10.1016/0003-9861(68)90654-1)
- Hernández-Pérez T, Gómez-García MDR, Valverde ME, Pares-López O (2020) *Capsicum annuum* (hot pepper): an ancient Latin-American crop with outstanding bioactive compounds and nutraceutical potential. A review. *Compr Rev Food Sci Food Saf* 19:2972–2993. <https://doi.org/10.1111/1541-4337.12634>
- Jang SW, Yoo MH, Hong WJ, Kim YJ, Lee EJ, Jung KH (2020) Re-analysis of 16S amplicon sequencing data reveals soil microbial population shifts in rice fields under drought condition. *Rice* 13:44. <https://doi.org/10.1186/s12284-020-00403-6>
- Jing J, Cong W-F, Bezemer TM (2022) Legacies at work: plant–soil–microbiome interactions underpinning agricultural sustainability. *Trends in Plant Sci* 27:781–792. <https://doi.org/10.1016/j.tplants.2022.05.007>
- Kabir MY, Nambeesan SU, Bautista J, Díaz-Pérez JC (2021) Effect of irrigation level on plant growth, physiology and fruit yield and quality in bell pepper (*Capsicum annuum* L.). *Sci Hortic* 281:109902. <https://doi.org/10.1016/j.scienta.2021.109902>
- Kakouridis A, Hagen JA, Kan MP, Mambelli S, Feldman LJ, Herman DJ, Weber PK, Pett-Ridge J, Firestone MK (2022) Routes to roots: direct evidence of water transport by arbuscular mycorrhizal fungi to host plants. *New Phytol* 236:210–221. <https://doi.org/10.1111/nph.18281>
- Khazaei Z, Esmailpour B, Estaji A (2020) Ameliorative effects of ascorbic acid on tolerance to drought stress on pepper (*Capsicum annuum* L.) plants. *Physiol Mol Biol Plants* 26:1649–1662. <https://doi.org/10.1007/s12298-020-00846-7>
- Köljal U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B, Drenkhan T, Eberhardt U, Dueñas M, Grebenc T, Griffith GW, Hartmann M, Kirk PM, Kohout P, Larsson E, Lindahl BD, Lücking R, Martín MP, Matheny PB, Nguyen NH, Niskanen T, Oja J, Peay KG, Peintner U, Peterson M, Pöldmaa K, Saag L, Saar I, Schübler A, Scott JA, Senés C, Smith ME, Suija A, Taylor DL, Telleria MT, Weiss M, Larsson KH (2013) Towards a unified paradigm for sequence-based identification of fungi. *Mol Ecol* 22:5271–5277. <https://doi.org/10.1111/mec.12481>
- Li C, Li H, Yao T, Su M, Li J, Liu Z, Xin Y, Wang L, Chen J, Gun S (2020) Effects of microbial inoculation on enzyme activity, available nitrogen content, and bacterial succession during pig manure composting. *Bioresour Technol* 306:123167
- Liu CY, Zhang F, Zhang DJ, Srivastava AK, Wu Q-S, Zou YN (2018) Mycorrhiza stimulates root-hair growth and IAA synthesis and transport in trifoliate orange under drought stress. *Sci Rep* 8:1978. <https://doi.org/10.1038/s41598-018-20456-4>

- Liu W, Park SW (2021) 12-oxo-Phytodienoic acid: a fuse and/ or switch of plant growth and defense responses? *Front Plant Sci* 12:724079. <https://doi.org/10.3389/fpls.2021.724079>
- López-Ráez JA, Verhage A, Fernández I, García JM, Azcón-Aguilar C, Flors V, Pozo MJ (2010) Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. *J Exp Bot* 61(2589–2601):2589–2601. <https://doi.org/10.1093/jxb/erq089>
- Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:550. <https://doi.org/10.1186/s13059-014-0550-8>
- Lutz S, Bodenhausen N, Hess J, Valzano-Held A, Waelchli J, Deslandes-Hérolld G, Schlaeppi K, van der Heijden MAG (2023) Soil microbiome indicators can predict crop growth response to large-scale inoculation with arbuscular mycorrhizal fungi. *Nat Microbiol* 8:2277–2289. <https://doi.org/10.1038/s41564-023-01520-w>
- Maestro-Gaitán I, Granado-Rodríguez S, Redondo-Nieto M, Battaglia A, Poza-Viejo L, Matías J, Bolaños L, Reguera M (2023) Unveiling changes in rhizosphere-associated bacteria linked to the genotype and water stress in quinoa. *Microb Biotechnol* 16:2326–2344. <https://doi.org/10.1111/1751-7915.14337>
- Mahmood T, Rana RM, Ahmar S, Saeed S, Gulzar A, Khan MA, Wattoo FM, Wang X, Branca F, Mora-Poblete F, Mafra GS, Du X (2021) Effect of drought stress on capsaicin and antioxidant contents in pepper genotypes at reproductive stage. *Plants* 10:1286. <https://doi.org/10.3390/plants10071286>
- Marino G, Brunetti C, Tattini M, Romano A, Biasioli F, Tognetti R, Loreto F, Ferrini F, Centritto M (2017) Dissecting the role of isoprene and stress-related hormones (ABA and ethylene) in *Populus nigra* exposed to unequal root zone water stress. *Tree Physiol* 37:1637–1647. <https://doi.org/10.1093/treephys/tpx083>
- Masoumi Z, Haghighi M, Jalali SAH (2021) Flooding or drought which one is more offensive on pepper physiology and growth? *Mol Biol Rep* 48:4233–4245. <https://doi.org/10.1007/s11033-021-06437-3>
- McDonald D, Jiang Y, Balaban M, Cantrell K, Zhu Q, Gonzalez A, Morton JT, Nicolaou G, Parks DH, Karst SM, Albertsen M, Hugenholtz P, DeSantis T, Song SJ, Bartko A, Havulinna AS, Jousilahti P, Cheng S, Inouye M, Niiranen T, Jain M, Salomaa V, Lahti L, Mirarab S, Knight R (2023) Greengenes2 unifies microbial data in a single reference tree. *Nat Biotechnol* 1–4. <https://doi.org/10.1038/s41587-023-01845-1>
- Miranda FR, Gondim RS, Costa CAG (2006) Evapotranspiration and crop coefficients for tabasco pepper (*Capsicum frutescens* L.). *Agric Water Manag* 82:237–246. <https://doi.org/10.1016/j.agwat.2005.07.024>
- Mishra N, Jiang C, Chen L, Paul A, Chatterjee A, Shen G (2023) Achieving abiotic stress tolerance in plants through antioxidative defense mechanisms. *Front Plant Sci* 14:1110622. <https://doi.org/10.3389/fpls.2023.1110622>
- Muhammad I, Yang L, Ahmad S, Zeeshan M, Farooq S, Ali I, Khan A, Zhou XB (2022) Irrigation and nitrogen fertilization alter soil bacterial communities, soil enzyme activities, and nutrient availability in maize crop. *Front Microbiol* 13:833758. <https://doi.org/10.3389/fmicb.2022.833758>
- Munoz-Ucos J, Wilhelm RC, Buckley DH, Bauerle TL (2022) Drought legacy in rhizosphere bacterial communities alters subsequent plant performance. *Plant Soil* 471:443–461. <https://doi.org/10.1007/s11104-021-05227-x>
- Naseem H, Ahsan M, Shahid MA, Khan N (2018) Exopolysaccharides producing rhizobacteria and their role in plant growth and drought tolerance. *J Basic Microbiol* 58:1009–1022. <https://doi.org/10.1002/jobm.201800309>
- Nerva L, Chitarra W, Fila G, Lovat L, Gaiotti F (2023) Variability in stomatal adaptation to drought among grapevine cultivars: genotype-dependent responses. *Agriculture* 13:2186. <https://doi.org/10.3390/agriculture13122186>
- Nerva L, Giudice G, Quiroga G, Belfiore N, Lovat L, Perria R, Volpe MG, Moffa L, Sandrini M, Gaiotti F, Balestrini R, Chitarra W (2022) Mycorrhizal symbiosis balances root-stock-mediated growth-defence tradeoffs. *Biol Fertil Soils* 58:17–34. <https://doi.org/10.1007/s00374-021-01607-8>
- Nurzyńska-Wierdak R, Buczkowska H, Salata A (2021) Do AMF and irrigation regimes affect sweet pepper fruit quality under open field conditions? *Agronomy* 11:2349. <https://doi.org/10.3390/agronomy11112349>
- Olmstead R, Bohs L, Migid H, Santiago-Valentin E, Garcia V, Collier S (2008) Molecular phylogeny of the Solanaceae. *Taxon* 57:1159–1181. <https://doi.org/10.1002/tax.574010>
- Pal SC, Hossain MB, Mallick D, Bushra F, Abdullah SR, Dash PK, Das D (2024) Combined use of seaweed extract and arbuscular mycorrhizal fungi for alleviating salt stress in bell pepper (*Capsicum annuum* L.). *Sci Hortic* 325:112597. <https://doi.org/10.1016/j.scienta.2023.597>
- Philippot L, Chenu C, Kappler A, Rillig MC, Fierer N (2024) The interplay between microbial communities and soil properties. *Nat Rev Microbiol* 22:226–239. <https://doi.org/10.1038/s41579-023-00980-5>
- Rodríguez Padrón R, Guedes J, Swarowsky A, Nogueira C, Cerquera R, Díaz-Pérez J (2015) Supplemental irrigation levels in bell pepper under shade mesh and in open-field: crop coefficient, yield, fruit quality and water productivity. *Afr J Agric Res* 10:4117–4125. <https://doi.org/10.5897/AJAR2015.10341>
- Rosa-Martínez E, Bovy A, Plazas M, Tikunov Y, Prohens J, Pereira-Dias L (2023) Genetics and breeding of phenolic content in tomato, eggplant and pepper fruits. *Front Plant Sci* 14. <https://www.frontiersin.org/articles/10.3389/fpls.2023.1135237>
- Sandrini M, Nerva L, Sillo F, Balestrini R, Chitarra W, Zampieri E (2022) Abiotic stress and belowground microbiome: the potential of omics approaches. *Int J Mol Sci* 23:1091. <https://doi.org/10.3390/ijms23031091>
- Santos-Medellín C, Liechty Z, Edwards J, Nguyen B, Huang B, Weimer BC, Sundaresan V (2021) Prolonged drought imparts lasting compositional changes to the rice root microbiome. *Nat Plants* 7:1065–1077. <https://doi.org/10.1038/s41477-021-00967-1>
- Sbrana C, Agnolucci M, Avio L, Giovannini L, Palla M, Turini A, Giovannetti M (2022) Mycorrhizal symbionts and

- associated bacteria: potent allies to improve plant phosphorus availability and food security. *Front Microbiol* 12. <https://www.frontiersin.org/journals/microbiology/articles/10.3389/fmicb.2021.797381>
- Shalaby OA, El-Sayed Ramadan ME-S (2024) Mycorrhizal colonization and calcium spraying modulate physiological and antioxidant responses to improve pepper growth and yield under salinity stress. *Rhizosphere* 29:100852. <https://doi.org/10.1016/j.rhisph.2024.100852>
- Singleton VL, Orthofer R, Lamuela-Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* 299:152–178. [https://doi.org/10.1016/S0076-6879\(99\)99017-1](https://doi.org/10.1016/S0076-6879(99)99017-1)
- Sinsabaugh R, Saiya-Cork K, Long T, Osgood M, Neher D, Zak D, Norby R (2003) Soil microbial activity in a liquidambar plantation unresponsive to CO₂-driven increases in primary production. *Appl Soil Ecol* 24:263–271. [https://doi.org/10.1016/S0929-1393\(03\)00002-7](https://doi.org/10.1016/S0929-1393(03)00002-7)
- Soare R, Maria D, Babeanu C, Popescu M, Popescu A (2017) Nutritional value and antioxidant activities in fruit of some cultivars of pepper (*Capsicum annuum* L.). *J Agroalimnet Processes Technol* 23:217–222
- Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, Berbee ML, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, James TY, O'Donnell K, Roberson RW, Taylor TN, Uehling J, Vilgalys R, White MM, Stajich JE (2016) A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108:1028–1046. <https://doi.org/10.3852/16-042>
- Suntornsuk L, Gritsanapun W, Nilkamhank S, Paochom A (2002) Quantitation of vitamin C content in herbal juice using direct titration. *J Pharm Biomed Anal* 28:849–855. [https://doi.org/10.1016/S0731-7085\(01\)00661-6](https://doi.org/10.1016/S0731-7085(01)00661-6)
- Tang C, Sun B, Zeeshan M, Li J, Zhang X (2023) *Funneliformis mosseae*-induced changes of rhizosphere microbial community structure enhance *Capsicum annuum* L. plant growth and fruit yield. *Soil Sci Soc Am* 87:843–855. <https://doi.org/10.1002/saj2.20528>
- Torres N, Goicoechea N, Carmen Antolín M (2018) Influence of irrigation strategy and mycorrhizal inoculation on fruit quality in different clones of Tempranillo grown under elevated temperatures. *Agric Water Manag* 202:285–298. <https://doi.org/10.1016/j.agwat.2017.12.004>
- Trouvelot A (1986) Mesure du taux de mycorhization VA d'un système racinaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi- Pearson V, Gianinazzi S (eds) *Physiological and genetical aspects of mycorrhizae*. INRA, Paris, pp 217–221
- Türkmen Ö, Sensoy S, Demir S, Erdinc C (2008) Effects of two different AMF species on growth and nutrient content of pepper seedlings grown under moderate salt stress. *Afr J Biotechnol* 7:392–396
- Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Sci* 151:59–66. [https://doi.org/10.1016/S0168-9452\(99\)00197-1](https://doi.org/10.1016/S0168-9452(99)00197-1)
- Wang J, Fu W, Sun C, Cai S, Tang C (2022) *Funneliformis mosseae* inoculation enhances *Cucurbita pepo* L. plant growth and fruit yield by reshaping rhizosphere microbial community structure. *Diversity* 14:932. <https://doi.org/10.3390/d14110932>
- Williams A, de Vries FT (2020) Plant root exudation under drought: implications for ecosystem functioning. *New Phytol* 225:1899–1905. <https://doi.org/10.1111/nph.16223>
- Wu QS, Zou YN (2017) Arbuscular mycorrhizal fungi and tolerance of drought stress in plants. In: QS Wu (Ed.), *Arbuscular mycorrhizas and stress tolerance of plants*. Springer 25–41. https://doi.org/10.1007/978-981-10-4115-0_2
- Yasuda M, Dastogeer KMG, Sarkodee-Addo E, Tokiwa C, Isawa T, Shinozaki S, Okazaki S (2022) Impact of *Azospirillum* sp. B510 on the rhizosphere microbiome of rice under field conditions. *Agronomy* 12:1367. <https://doi.org/10.3390/agronomy12061367>
- Zhang J, Cook J, Nearing JT, Zhang J, Raudonis R, Glick BR, Langille MGI, Cheng Z (2021) Harnessing the plant microbiome to promote the growth of agricultural crops. *Microbiol Res* 245:126690. <https://doi.org/10.1016/j.micres.2020.126690>
- Zhang H, Sun X, Dai M (2022a) Improving crop drought resistance with plant growth regulators and rhizobacteria: mechanisms, applications, and perspectives. *Plant Commun* 3:100228. <https://doi.org/10.1016/j.xplc.2021.100228>
- Zhang L, Zhou J, George TS, Limpens E, Feng G (2022b) Arbuscular mycorrhizal fungi conducting the hyphosphere bacterial orchestra. *Trends Plant Sci* 27:402–411. <https://doi.org/10.1016/j.tplants.2021.10.008>
- Zheng FL, Liang SM, Chu XN, Yang YL, Wu QS (2020) Mycorrhizal fungi enhance flooding tolerance of peach through inducing proline accumulation and improving root architecture. *Plant Soil Environ* 66:624–631. <https://doi.org/10.17221/520/2020-PSE>
- Zhishen J, Mengcheng T, Jianming W (1999) The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem* 64:555–559. [https://doi.org/10.1016/S0308-8146\(98\)00102-2](https://doi.org/10.1016/S0308-8146(98)00102-2)
- Zhou Y, Yi S, Zang Y, Yao Q, Zhu H (2021) The Predatory Myxobacterium *Citricoccus inhibens* gen. nov. sp. nov. showed antifungal activity and bacteriolytic property against phytopathogens. *Microorganisms* 9:2137. <https://doi.org/10.3390/microorganisms9102137>
- Zia R, Nawaz MS, Siddique MJ, Hakim S, Imran A (2021) Plant survival under drought stress: Implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. *Microbiol Res* 242:126626. <https://doi.org/10.1016/j.micres.2020.126626>
- Zou YN, Wu QS, Kuča K (2021) Unravelling the role of arbuscular mycorrhizal fungi in mitigating the oxidative burst of plants under drought stress. *Plant Biol* 23:50–57. <https://doi.org/10.1111/plb.13161>

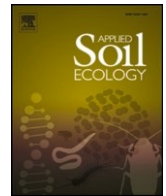
Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Chapter II

This chapter is the result of a work conducted in collaboration with the research group of Prof. Bacem Mnasri. The work has been published on Applied Soil Ecology in 2025. The objective of this two-year field study was to assess the impact of PGPB inoculation on two different pea varieties.

The used varieties were Merveille de Kelvedon and Douce de Provence, both inoculated with a *Rhizobium* strain, with *Rhizobium* and *Erwinia* strains, as well as not-inoculated plants acting as control. Both *Rhizobium* and *Erwinia* strains were previously isolated from Tunisia (Ilahi et al., 2024, 2021). The aim of the experiment was to assess the effect of the different inoculation conditions on pea physiological and productivity parameters, as well as the effect on the natural microbial communities of the rhizosphere, including both AMF and bacteria.

This study demonstrated the potential of targeted bacterial inoculations isolated from the same geographical area in improving pea cultivation in semi-arid environments. Additionally, positive interactions were detected among the natural soil-borne microorganisms under PGPB inoculation, suggesting that PGPB inoculation may trigger also specific microorganism-microorganism interaction that can have a positive effect on plant growth. The findings emphasized microbial inoculants as a sustainable agricultural practice for such regions, while also contributing to a better understanding of their influence on native bacterial and AM fungal communities.



Research paper

Impact of bacterial inoculations on *Pisum sativum* L. biometric parameters and associated bacterial and AM fungal communities under semi-arid field conditions in Tunisia

Houda Ilahi ^{a,b,1}, Alice Calvo ^{c,d,1}, Sana Dhane ^e, Mustapha Missbah El Idrissi ^f,
Lahcen Ouahmane ^g, Mohamed Najib Alfeddy ^h, Thomas Reitz ^{i,j}, Fabiano Sillo ^{c,*},
Raffaella Balestrini ^k, Bacem Mnasri ^b



^a Faculty of Sciences of Tunis, University Tunis El Manar, 2092 Tunis, Tunisia

^b Laboratory of Legumes and Sustainable Agrosystems, Centre of Biotechnology of Borj-Cédria, BP, 901 Hammam-lif, 2050, Tunisia

^c National Research Council of Italy, Institute for Sustainable Plant Protection (IPSP), Torino, Italy

^d University of Sassari, Department of Agriculture and Desertification Research Centre, Sassari, Italy

^e University of Carthage, National Agronomic Institute of Tunisia, Horticultural Sciences Laboratory, LR13AGR01, Tunisia

^f Faculty of Sciences, Centre de Biotechnologies Végétale et Microbienne, Biodiversité et Environnement, Mohammed V University in Rabat, Morocco

^g Laboratory of Microbial Biotechnologies Agrosciences and Environment, Cadi Ayyad University, Marrakesh 40000, Morocco

^h Phytobacteriology Laboratory Plant Protection Research, Unit CRRR Marrakesh National Institute for Agronomical Research, Marrakesh 40000, Morocco

ⁱ Helmholtz Centre for Environmental Research GmbH – UFZ, Department of Soil Ecology, Halle, Germany

^j German Centre for Integrative Biodiversity Research (iDiv), Halle-Jena-Leipzig, Germany

^k National Research Council of Italy, Institute of Biosciences and Bioresources (IBBR), Bari, Italy

ARTICLE INFO

ABSTRACT

Keywords:

Pea
Arbuscular mycorrhizal fungi
PGPB
Plant-microbe interactions
Rhizobium
Erwinia

In Mediterranean agroecosystems, pea (*Pisum sativum* L.) is one important crop due to its nutritional benefits and high protein content. However, soil nutrient availability and soil health are known to affect pea productivity, especially under arid and semi-arid conditions. Currently, the use of plant growth-promoting bacteria (PGPB) may represent a bio-based tool to improve pea productivity in drought-affected areas. Nevertheless, there is limited knowledge on how PGPB inoculations in field could impact native communities of bacteria and arbuscular mycorrhizal fungi (AMF) in these areas. Here, a two-year field study in Tunisia was established to evaluate the effects of inoculating two pea varieties with three strains of potential PGPB, including *Rhizobium laguerreae* and two strains of *Erwinia* sp., on agronomic performance and soil microbial communities. Inoculations improved productivity and all measured biometric parameters, with the treatment including a consortia of the three strains showing the highest benefits. Metabarcoding analysis showed an increased bacterial and AM fungal diversity in soil of inoculated plants. Additionally, specific AMF-bacterial associations were identified, suggesting a synergistic role in enhancing soil health and pea growth. Overall, this study highlights the potential of targeted bacterial inoculations to improve pea performance under semi-arid environments by exploiting beneficial plant-microbe interactions. These results support the use of microbial inoculants as a sustainable agricultural practice in semi-arid areas, also improving the understanding of their impact on native bacterial and AM fungal communities.

1. Introduction

In the Mediterranean region, leguminous species are a key component of agroecosystems (Maxted and Bennett, 2001) and, among them, pea (*Pisum sativum* L.) represents one of the most cultivated (Missbah El

Idrissi et al., 2020). Pea, known for its substantial nutritional and health benefits (Ilahi et al., 2021), is an important leguminous crop especially valued for its high protein content in developing countries (Lu et al., 2019; Ejaz et al., 2020). Additionally, it is considered a functional food due to the presence of bioactive compounds like phenolic ones, which

* Corresponding author.

¹ These authors contributed equally as first authors.

<https://doi.org/10.1016/j.apsoil.2024.105757>

Received 2 August 2024; Received in revised form 8 November 2024; Accepted 14 November 2024

Available online 25 November 2024

0929-1393/© 2024 Elsevier B.V. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

have health-promoting properties, i.e., antioxidant and anti-inflammatory (Nazir et al., 2020). However, the availability of nitrogen (N) and phosphorus (P) nutrients is crucial for the accumulation of phenolic compounds in plant tissues and plays an important role in determining the antioxidative status of pea plants (Stewart et al., 2001). Due to pea low tolerance to biotic and abiotic stresses, major problems in its cultivation are represented by yield instability in relation to site and season variability (Rubiales et al., 2014). As a result, pea cultivation is particularly limited in the Mediterranean Basin and recently released pea cultivars are poorly adapted to Mediterranean environments (Rubiales et al., 2021).

Tunisia is currently considered as a climate change hotspot. In the agricultural systems the damages and losses for crops resulting from climatic issues are increasingly reported (Young et al., 2012). In the last years, the combined use of chemical fertilizers and drought have led to a decreased agricultural production (Radhouane, 2018; Besser et al., 2021). In this scenario, the need for the development and application of sustainable alternatives to chemical fertilizers, as the use of microbial-based solutions, has been rising. Currently, in sustainable agricultural management, alternatives to the chemical fertilization by using environmental-friendly practices to enhance crop productivity are considered as promising tools (Yadav, 2020). Sustainable approaches include the use of bio-based solutions relying on the interactions between plants and beneficial microorganisms, including plant growth-promoting bacteria (PGPB) and arbuscular mycorrhizal fungi (AMF) (Hakim et al., 2021; Balestrini et al., 2024). These interactions play a crucial role in enhancing plant nutrient uptake and soil fertility, while also influencing the surrounding microbial communities in the rhizosphere (Trivedi et al., 2020).

Plant growth-promoting bacteria are a group of bacteria able to improve plant growth and fitness without establishing symbiotic relationship with the host roots (Mantelin and Touraine, 2004). It has been demonstrated that they can be used as biofertilizers to improve crop productivity and resilience, particularly under challenging environmental constraints such as salinity and aridity (Gritli et al., 2023; Ilahi et al., 2024). Furthermore, some PGPB are known to enhance biological nitrogen fixation (Mahmoud et al., 2023), thereby reducing reliance on N fertilizers and contributing to soil health. Plant growth-promoting bacteria are a heterogeneous group of bacteria that include several different genera e.g., *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, and *Variovorax* (Arora et al., 2016).

Among PGPB, strains of *Erwinia* sp. have proved to enhance crop growth and resistance to abiotic stresses (Ilahi et al., 2024) via different mechanisms i.e., N fixation, phosphate solubilization and siderophore production (Tozlu et al., 2012), as well as to improve plant tolerance to salt stress (Singh et al., 2018). Moreover, strains of *Erwinia* sp. are known to be able to improve plant tolerance to drought producing bacterial 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, volatiles, antioxidant compounds, cytokinin and indole-3-acetic acid (IAA) (Kasotia et al., 2016), therefore representing a sustainable approach in arid and semi-arid environments to boost crop productivity.

Differentially from PGPB, rhizobia establish symbiotic relationship with plant through the formation of root nodules (Mantelin and Touraine, 2004). Rhizobia are a group of bacteria belonging to Alphaproteobacteria and Betaproteobacteria classes, they are able to produce N_2 -fixing nodules (Poole et al., 2018), and they are known for their ability in establishing root symbiosis with leguminous plants e.g., pea (Ejaz et al., 2020). Some rhizobia species, identified as beneficial microorganisms, include *Rhizobium leguminosarum*, *R. pisi*, *R. fabae*, *R. laguerreae*, *R. bangladeshense*, *R. lentis*, *R. binae* and *R. anhuiense* (Ilahi et al., 2021). In particular, the species *R. laguerreae* has been identified as the most prevalent among *Rhizobium* species in Tunisian soils (Ilahi et al., 2021). Biological N fixation, mediated by rhizobia (Lindström and Mousavi, 2020), has been reported to supply >50 % of the N required by pea plants, contributing approximately 25 kg of N per hectare to the soil for the subsequent crop (Ejaz et al., 2020). Despite a slightly lower effectiveness compared to mineral N fertilization, the importance of selected rhizobium inoculation in increasing leguminous crop yields has

been confirmed (dos Santos Sousa et al., 2022), suggesting their use as bio-based alternatives to inorganic fertilization.

Similarly to rhizobia, arbuscular mycorrhizal fungi (AMF) are symbiotic organisms able to establish strong relationships with plant roots, enhancing nutrient and water uptake (Pasquini et al., 2023). For this reason, they represent a promising tool in improving plant tolerance to water deficit conditions and lack of nutrients in the soil (George and Ray, 2023). Moreover, the symbiotic relationship between plant roots and AMF fungal hyphae leads to the recruitment of specialized microorganisms in the so-called mycorrhizosphere, i.e., the soil area influenced by roots colonized by AMF (Giovannini et al., 2020; Scheublin et al., 2010; Williams et al., 2024). This microbial recruitment has been reported to play a positive role in plant development, nutrient absorption, as well as tolerance to abiotic and biotic stresses (Philippot et al., 2023). In arid and semi-arid regions, accelerated desertification has led to soil degradation and reduced vegetation cover, negatively affecting plant growth (Mahmoudi et al., 2019). In these regions, symbiotic relationships between plants and AMF are vital for sustaining plant growth, particularly in protected Mediterranean conservation areas (Mahmoudi et al., 2020). Recent studies have been performed to explore AM fungal distribution in natural soils, by evaluating the relative abundances of different genera of virtual taxa (VTXs) of AMF in arid and semi-arid environments (El Hazzat et al., 2018; Dalli et al., 2020; Gritli et al., 2023; Noreen et al., 2023). According to Gritli et al. (2023), most of the identified AM fungal genera, associated to *Lathyrus cicera* roots in soils of northern Tunisia, were represented by *Glomus*, *Claroideoglomus*, *Diversispora*, *Paraglomus*, and *Scutellospora*. In comparison, a study in western Algeria showed *Glomus* and *Acaulospora* as dominant genera associated with carob trees (Dalli et al., 2020). Moreover, *Glomus* has been detected as the most representative AM fungal genus in Morocco and Pakistan in chickpea, beans, and other legumes (El Hazzat et al., 2018; Noreen et al., 2023). Nevertheless, knowledge about natural plant-AM fungal interactions in arid ecosystems, including Tunisia, is still limited. Moreover, the effects of the use of bacterial inoculants in field on fungal communities have been shown to be variable effects with no clear results demonstrated (Trabelsi and Mhamdi, 2013).

The aim of this two-year field study was to evaluate the beneficial effects of inoculation with one strain of *Rhizobium laguerreae* (Ilahi et al., 2021) and two strains of *Erwinia* sp., previously characterized as PGPB (Ilahi et al., 2024), on the biometric parameters and plant estimated productivity of two *P. sativum* varieties grown in fields in Tunisia. The second focus of this study was to examine the impact of the bacterial inoculations on pea-associated microbial taxa, focusing on AM fungal and bacterial communities in rhizospheric soil. It has been documented so far that the composition of the rhizosphere microbial community is affected by exudates released by plant species/variety and soil type (Gregory, 2006), thus allowing us to understand the dynamics of the rhizosphere compartment as influenced by both roots and bulk soil. Revealing the variety dependent responses to microbial inoculations will allow to explore the potential of microbial inocula to enhance pea plant growth and productivity by exploiting the synergistic interactions between specific bacterial and AM fungal communities in the soil, thus promoting sustainable agriculture practices.

2. Materials and methods

2.1. Experimental design

The experiment was conducted at the experimental site of the Higher School of Agriculture of Mateur, located in the governorate of Bizerte, delegation of Mateur, approximately 70 km northwest of Tunis. The site is located at a latitude of 37°37'7.017"N, longitude of 9°37'13.429"E and has an elevation of 19 m above sea level. The experiment was conducted

in the first year from December 1, 2020 to April 29, 2021 and for the second year from December 1, 2021 to April 29, 2022. The used plant material consisted of two varieties of pea (*Pisum sativum* L.), namely the very early variety “Douce de Provence” (DP) and the early variety “Merveille de Kelvedon” (MK). These two varieties were introduced from France and are now the two most cultivated pea varieties in Tunisia (Barhoumi et al., 2023), representing a standard choice for pea sustainable cultivation in this area. The experiment comprised a total area of 350 m², partitioned into two equally sized field parcels of 175 m² for each pea variety. These parcels were delineated with a 2-m margin to minimize cross-contamination and ensure distinct separation of the experimental factors (Fig. S1). Each parcel contained three systematically distributed plot replicates dedicated to each of the three different treatments: i) inoculation with a *Rhizobium* strain (Rh), ii) inoculation with *Rhizobium* and *Erwinia* strains (PG Rh) and iii) not-inoculated control (CTRL), for a total of nine parcels, each of 14 m² and arranged in eight rows spaced 0.5 m apart (Fig. S1). The allocation of pea varieties in the plots was determined by their maturation rates: DP was planted in the first plot due to its earlier maturity, while MK was assigned to the second plot. The cultivation protocol initiated with land preparation, plowing to a depth of 20 cm followed by two subsequent passes with an offset implement, to ensure soil aeration and preparation. Planting rows were manually established, followed by the hand sowing of seeds at prescribed densities of 20 seeds/m² for *P. sativum*. Organic cultivation practices were employed, with no chemical treatments or fertilizers application, and manual weeding using a hoe. No irrigation practices were applied to pea plants, i.e., they relied uniquely on rainfall variability. In the experimental field, a crop rotation, comprising pea, faba bean and durum wheat, was applied before 2021. Climatic data, including monthly rainfall (mm) as well as monthly averages of humidity (%) and temperature (°C) have been collected for the two experimental periods in Mateur from December 2020 to May 2021 and from December 2021 to May 2022 and are summarized in Fig. S2. Daylight hours have been also recorded showing that the average daylight hours for both year are, from December to May, 09:40 h, 10:10 h, 10:50 h, 11:51 h, 13:30 h, and 14:08 h, respectively.

2.2. Bacterial inoculation

To obtain rhizobia and *Erwinia* inocula, three bacterial strains were sourced from the laboratory of legumes and sustainable Agrosystems (L2AD) in the Centre of Biotechnology of Borj Cedria (CBBC, Tunisia). The native strains 25PS6 of *Rhizobium laguerreae* sv. *viciae* (Ilahi et al., 2021) was used in Rh treatment. In addition to strain 25PS6, strains 12PS6 and 13PS9 of *Erwinia* (Ilahi et al., 2024) were used in PG Rh treatments. Each strain was grown separately to the late exponential phase in yeast-extract-mannitol broth (Vincent, 1970) and then diluted at 1:10 using 1 L of inoculum in 9 L of sterile water for a final concentration of 10⁹ CFU/mL. Each parcel of the inoculated conditions (Rh and PG Rh ones) received 10 L of the bacterial solution at seedling phase, providing approximately 5 mL of the diluted bacterial inoculum to each seedling.

2.3. Biometric parameters and nitrate content in pea leaves

At maturity, i.e., five months after the beginning of the experiment, three plants for each sub-plots were sampled for biometric parameters evaluation, for a total of nine plants for each treatment. Analyzed biometric parameters were shoot length (cm), shoot dry weight (g), plant nitrate content (ppm), pod number (n), pod weight (g), root dry weight (g) and root nodule number (n) of both varieties. For dry weight evaluation, the plants harvested at maturity were weighed, and then dissected into the three analyzed components, including shoots, pods and roots for individual weighing. Pod weight was evaluated as the average weight of 100 pods. After drying the plant material in an oven at 60 °C for three days, the dry weight was recorded. Concerning nitrate content analysis, the middle part of each leaf was grounded using a ceramic mortar and the obtained liquid matrix was used to assess nitrate

quantification using the “nitrate check” device Horiba LAQUAtwin NO3–11 Compact Nitrate Ion Meters (AllCaT Instruments, BP 32025, F13845, Vitrolles Cedex France). At root level, nodule number was assessed by count.

2.4. Soil sampling

Soils samples were collected via drilling at 10–20 cm depth before the beginning of the experiment for the assessment of soil physico-chemical characteristics. For this step, 1 kg of soil was collected from three random points at the experimental site and average data values were calculated for different parameters, including texture (clay, %, loam, % and sand, %), pH, exchangeable Na, Mg, Ca and K (mg/g, mg/L, mg/g and mg/L, respectively), assimilable P (μg/g), electrical conductivity (EC, mS/cm), soil organic matter content (SOM, %) and total nitrogen (N, %). Analyses were conducted at the Specialized Unit for Research Support and Technological Transfer at the Biotechnology Center of Borj-Cédria as described by Gritli et al., 2023. For metabarcoding analysis, rhizospheric soil was sampled from each parcel at the end of each growing season. Briefly, four/five soil samples were sampled in each parcel and merged to obtain one pooled sample for each treatment of approximately 250 g. All samples were then stored at -80 °C.

2.5. Statistical analysis

Statistical analysis was conducted with R software v. 4.3.2 on the different biometric parameters i.e., shoot length (cm), shoot dry weight (g), plant nitrate content (ppm), pod number (n) and weight (g), root dry weight (g) and root nodule number (n). Three-way ANOVA has been performed to assess the effects of factors “year”, “treatment”, and “variety”, as well as their interaction. Additionally, for each variety, data have been analyzed considering the two years of production (2021 and 2022) separately with two-way ANOVA, considering as factors the “treatment”, the “variety” and their interaction. Post-hoc analysis was performed through Tukey HSD Test with significance level set to $p \leq 0.05$. Heatmaps were produced with R package “pheatmap” to investigate the main AM fungal genera and, separately, the main bacterial genera associated to the different treatments in both years. Moreover, a correlation analysis between the presence of AMF and the concurrent presence of bacteria in the different treatments across both years has been performed using Pearson method, with thresholds set at $p \leq 0.05$ and R -square at 0.75.

2.6. Metabarcoding

Total DNA extraction was performed on 18 samples, corresponding to three replicates for each treatment for each year, by using the DNeasy PowerSoil Pro Kit. The 16S rRNA gene V4 region was amplified using the primers P5_8N_515F and P5_7N_515F along with P7_2N_806R and P7_1N_806R (Caporaso et al., 2011; Moll et al., 2018). For the fungal ITS2 regions, amplification followed the protocol outlined by Prada-Salcedo et al. (2021), utilizing primers P5–5/6 N-ITS4 (N{5,6} TCCTCCGCTTATTGATATGC) and P7–3/4 N-ITS7 (N{3,4} GTGART-CATCGAATCTTTG). The AMF SSU regions were amplified using a nested PCR approach (Wahdan et al., 2021) with primers GLOMERWTO (CGAGDWTCATTCAAATTTCTGCCC) and GLOMER1536 (AATARTTG-CAATGCTCTATCCCCA), followed by NS31 (TTGGAGGG-CAAGTCTGGTGCC) and AML2 (GAACCCAAACACTTTGGTTTCC). Each sample and target region underwent PCR amplification in triplicate (using 40 ng DNA template per PCR reaction) with Kapa HiFi polymerase (KapaBiosystems, Boston, USA). The PCR-triplicates amplicons were pooled and purified with AmpPure XP Beads, then indexed with an additional PCR using Illumina Nextera XT v2 index primers, followed by

another purification with AmpPure XP Beads. The concentration of indexed and purified PCR products was measured with a NanoDrop ND-8000 spectrophotometer. DNA from fungal and prokaryotic amplicons was pooled equimolarly. Exact concentrations of the final pools were

determined using a Qubit dsDNA-HS Assay, and fragment length and quality were further verified with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The combined prokaryotic and fungal amplicon pools were prepared for Illumina MiSeq paired-end

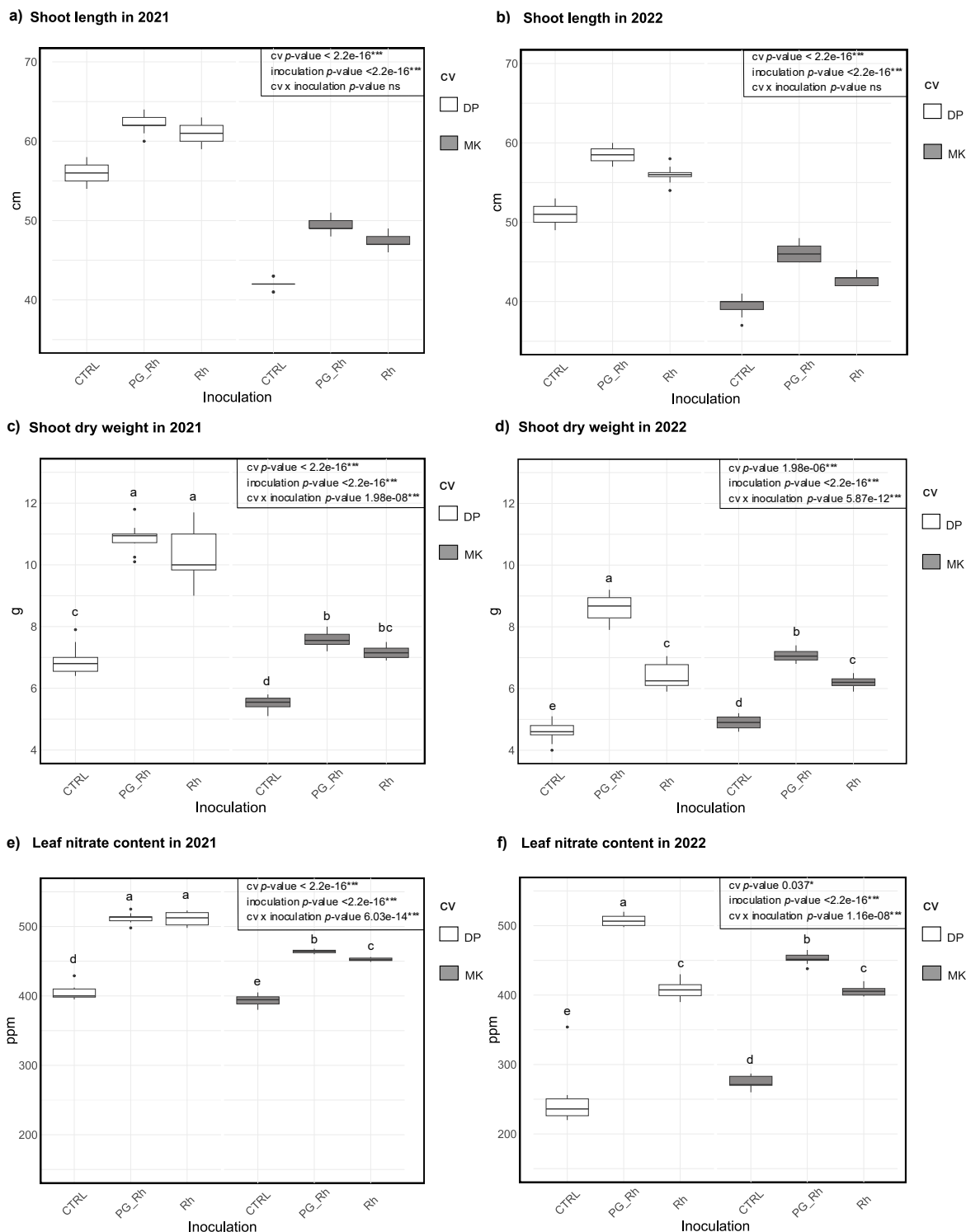


Fig. 1. Shoot biometric parameters of pea plants. a-b) Shoot length (cm), c-d) shoot dry weight (g) and e-f) leaf nitrate content (ppm) of DP and MK varieties in 2021 and 2022 subjected to the three different inoculation conditions ($n = 9$). ANOVA p -values are included (cv = cultivar, inoculation = inoculation condition, cv x inoculation = interaction). Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not -inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

sequencing. Sample libraries and the control library were diluted and denatured according to the MiSeq Illumina kit instructions and loaded onto an Illumina MiSeq flow cell for paired-end sequencing. Raw reads were analyzed through QIIME 2 pipeline (Bolyen et al., 2019; Estaki et al., 2020). Sequences trimming was performed with cutadapt v3.4. This step was followed by denoising through dada2 v2021.8.0 and the final assembling into Amplicon Sequence Variants (ASVs). The Maarjam (Opik et al., 2010) and Silva v. 138–99 (Quast et al., 2013) databases for AMF and bacteria, respectively, were used to obtain the taxonomic classification of each ASV. In both cases the output was elaborated to obtain a relative abundance (%) of each ASV in the total amounts of the entire sample. Raw reads from metabarcoding of soil samples were submitted to NCBI SRA database under BioProject ID PRJNA1141918. Two ASV tables were produced for the years 2021 and 2022 for AMF and bacteria, respectively (Tables S1–2). These ASV tables were used as input for Microbiome Analyst (Dhariwal et al., 2017; Chong et al., 2020) for taxon abundance visualization and diversity calculation. Diversity within samples i.e., alpha diversity was calculated with Chao1 and Shannon indexes, while diversity among samples i.e., beta diversity was calculated with Bray-Curtis index. The analysis was performed at Feature-level in both cases and beta diversity was reported in a two-dimensional principal coordinates analysis. Taxon abundance was

visualized for each year through Stacked Bar plot at family and order level for AMF and bacteria, respectively. Core analysis was performed to investigate the main taxa composition of each year and of each variety at order level. By using Microbiome Analyst, a Linear Discriminant Analysis Effect Size (LEfSe) analysis was performed, considering the two years together, to assess taxa relative abundance in each varieties and in inoculated and not-inoculated samples, as well as the interaction among these factors at all taxonomic level (feature level), to identify the most discriminant feature among the different conditions.

Additionally, differential abundance analysis of microbial taxa at genus level was conducted using the Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC; Lin and Peddada, 2020) embedded in QIIME2 (Estaki et al., 2020) to assess taxa showing changes between inoculated versus not-inoculated conditions. Relative abundances of bacterial and AMF genera in inoculated treatments, considering the two years together, were compared to control (not-inoculated).

The command “qiime composition ancombc” was used by setting [—p-formula “condition”], [—p-reference-levels “condition::not_inoculated”] and [—p-prv-cut 20].

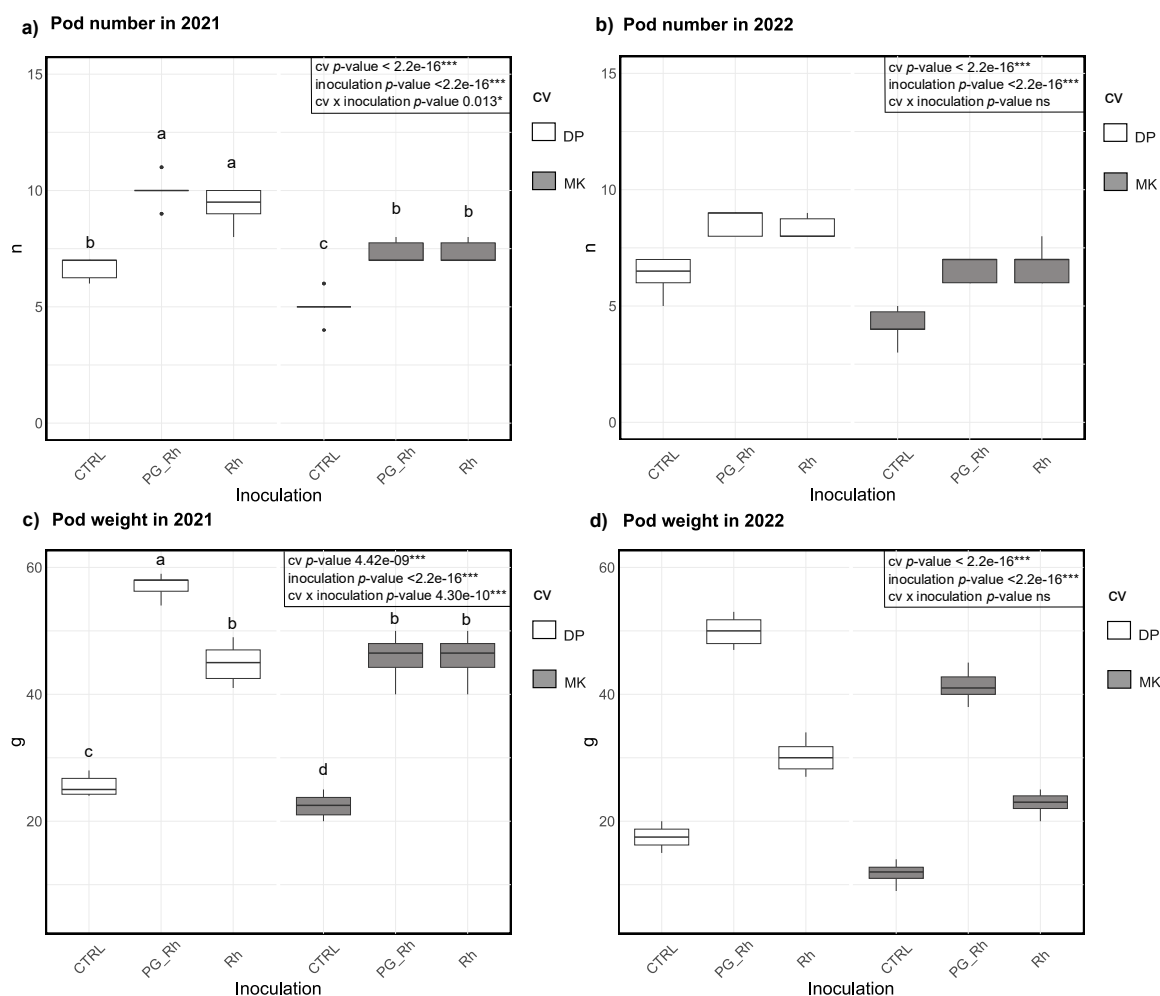


Fig. 2. Pea pod production. a-b) Pod number (n) and c-d) pod weight (g) of DP and MK varieties in 2021 and 2022 subjected to the three different inoculation conditions (n = 9). ANOVA p-values are included (cv = cultivar, inoculation = inoculation condition, cv x inoculation = interaction). Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

3. Results

3.1. Biometric parameters

All the analyzed biometric parameters including shoot length, shoot dry weight, nitrate content, pod number, pod weight, root dry weight and root nodule number were significantly affected by the factor “year” (Table S3). Overall, the year 2021 was characterized by greater plant biometric parameters and a higher production compared to the year 2022 (Table S3). In both years significant differences between varieties and among treatments were observed (Figs. 1–3). Specifically, all considered biometric parameters were significantly higher in DP variety plants compared to MK variety and a significant reduction was highlighted comparing CTRL to Rh and to PG Rh treatment in both pea cultivars. The PG Rh treatment had a significant effect on all the biometric parameters (Figs. 1–3). Plants of DP variety subjected to PG Rh showed the highest values compared to all the other treatments (Figs. 1–3).

3.2. Soil chemical-physical properties

The soil was classified as loamy soil, with a pH close to neutrality (7.43). Assimilable P (89.56 $\mu\text{g/g}$) was comparable to that reported in Hachana et al. (2021). The SOM % was relatively high (8.37 %). On the other hand, the total N % was relatively low (0.22 %). The exchangeable Na level of 22.06 mg/g was relatively high, as also an EC of 3.23 mS/cm suggested moderate salinity condition. Detailed information are reported in Table S4.

3.3. Soil AM fungal community

The total number of AM fungal reads obtained from Mateur soils was 599,119, of which 271,518 were identified in 2021 and 327,601 in 2022. Approximately 38.5 % and 35.5 % reads of 2021 and 2022, respectively, were assigned at family level, and a total of 59 taxa at VTX level were identified (Table S1). The core of AM fungal community of 2021 was represented by orders Glomerales, Paraglomerales, Diversisporales and Archaeosporales. Despite the presence of a common core, the AM fungal community was characterized by large taxon diversity

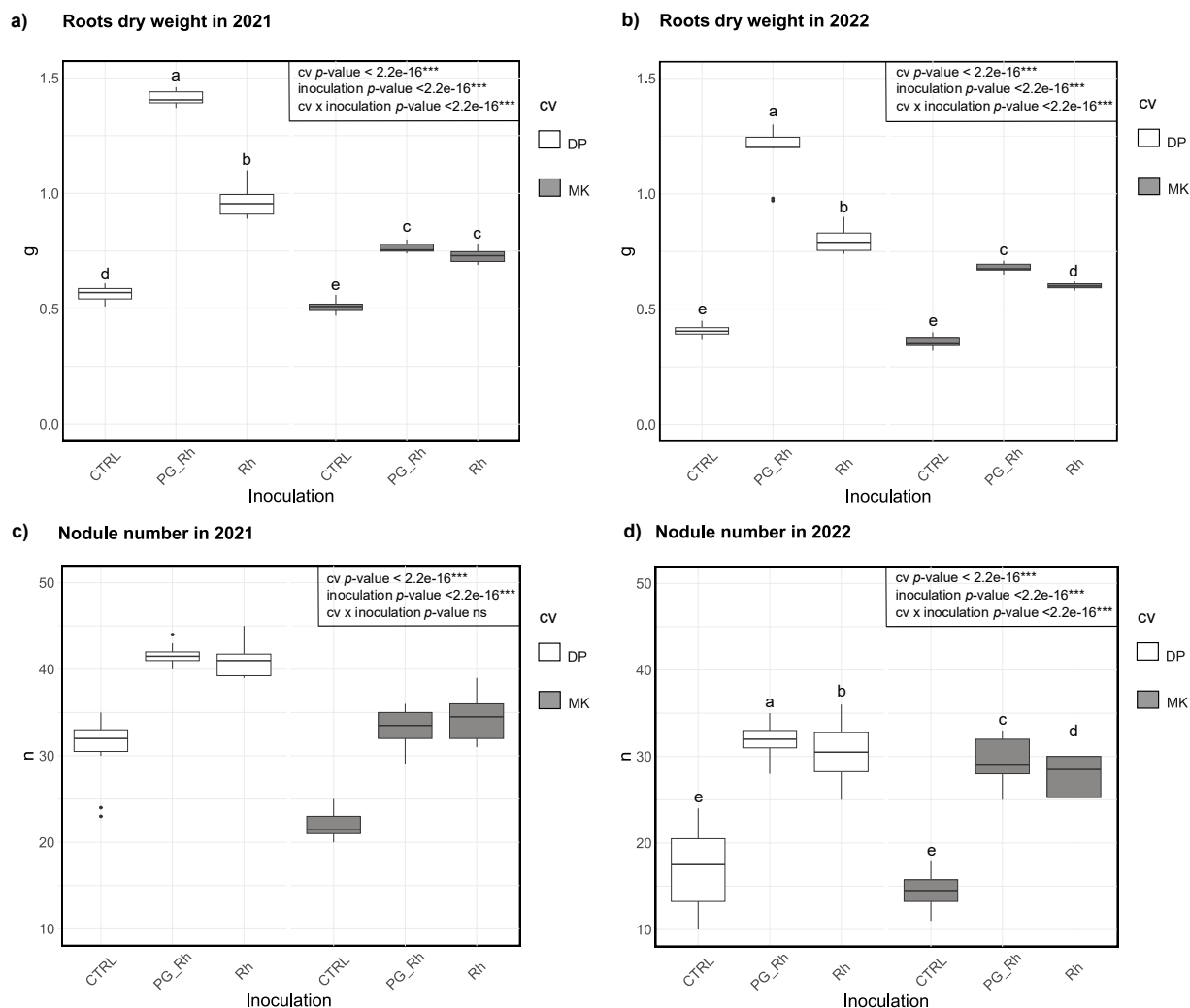


Fig. 3. Root biometric parameters of pea plants. a-b) Root dry weight (g) and c-d) root nodules number (n) of DP and MK varieties in 2021 and 2022 subjected to the three different inoculation conditions (n = 9). ANOVA *p*-values are included (cv = cultivar, inoculation = inoculation condition, cv x inoculation = interaction). Ns, *, **, ***: not significant or significant at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively. Different letters showed significant differences according to Tukey HSD test ($p \leq 0.05$), considering the associated significant source of variance in year 2021 and 2022. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not - inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

and abundance among treatments. Differences in taxon abundance between the two varieties (DP and MK) were evaluated by estimation of alpha diversity, that resulted significantly different with Chao1 index (p -value 0.017, Fig. 4), while not with Shannon index (p -value >0.05). In particular, Paraglomerales order was more abundant in DP, while Archaeosporales in MK. Moreover, differences in beta diversity were highlighted also considering the factors “variety” and “inoculation” (p -value 0.001 and p -value 0.003, respectively, Fig. S3a-b). At family level (Fig. 4), DP variety was associated to a higher abundance of Diversisporaceae and Paraglomeraceae, while MK to Claroidoglomeraceae, Acaulosporaceae and Archaeosporaceae. The family Claroideoglomeraceae, even if detected also in DP, showed a decrease trend in 2022 compared to 2021, probably related to different climatic conditions between the two growing seasons. The year 2022 was in fact dryer than 2021, as it was characterized by overall higher temperatures and lower amount of rainfall (Fig. S2). The decrease of Claroideoglomeraceae may be linked to their suggested sensibility to drought, as previously reported (Mao et al., 2024). Abundance analysis highlighted the presence of different VTXs associated to each treatment (Fig. 5a). In particular, *Claroidoglomus* sp. was associated to DP CTRL and MK PG Rh, *Diversispora* sp. to DP PG Rh and MK CTRL, while the highest abundance of *Archaeospora* sp. was recorded under MK Rh.

The core of the AM fungal community in 2022 was represented by orders Paraglomerales, Diversisporales and Glomerales. As for 2021, a significant difference was found for alpha diversity between DP and MK with Chao1 index (p -value 0.014, Fig. 4), but not using the Shannon index (p -value >0.05), with MK variety associated to a higher taxa abundance compared to DP. Beta diversity highlighted a significant difference between the two varieties (p -value 0.001, Fig. S3c), but not considering the “inoculation” factor (Fig. S3d). Specifically, MK variety showed a higher presence of Archaeosporales compared to DP one variety. Families Claroidoglomeraceae, Archaeosporaceae and Acaulosporaceae were more abundant in MK variety, while in DP a higher abundance of Diversisporaceae and Paraglomeraceae was recorded (Fig. 4). Abundance analysis among AM fungal VTXs and the different treatments highlighted that *Paraglomus* sp. was associated to DP CTRL, DP Rh and MK PG Rh, *Diversispora* sp. to all DP samples and MK CTRL, and *Archaeospora* sp. to MK PG Rh (Fig. 5b).

Considering both years, LEfSe analysis (Table S5) revealed the presence of specific AMF taxa associated to each pea variety at family level. Specifically, Acaulosporaceae, Gigasporaceae, Entrophosporaceae and Archaeosporaceae were associated to MK variety, while Paraglomeraceae were found associated to DP one. A further analysis has been conducted to assess a difference in taxa association and abundance between inoculated (Rh and PG Rh samples) and not-inoculated (CTRL) samples inside each variety samples. While in DP variety no significant differences were detected, in MK variety, inoculated samples were associated with a higher abundance of Acaulosporaceae taxa and not-inoculated ones were enriched in Diversisporaceae and Glomeraceae (Table S5). The ANCOM-BC method allowed to identify several taxa (genus level) in AMF communities showing greater abundance in inoculated treatments compared to not-inoculated ones. Among AMF taxa, the genus *Acaulospora* showed significant negative Log Fold Changes (LFCs) in inoculated conditions (Fig. 5c). On the other hand, positive LFCs were observed for genera including *Archaeospora*, *Scutellospora*, *Ambispora*, *Racocetra*, indicating an increase under the inoculated treatments (Fig. 5c).

3.4. Soil bacterial community

Bacterial reads assigned at least at order level accounted for the 94.5 % and 94.0 % of the total obtained reads from 2021 and 2022, respectively. The core microbiome of 2021 was characterized by orders Solirubrobacterales, Bacillales, Nitrososphaerales, Rhizobiales and Burkholderiales. Bacterial communities of 2021 did not show statistical

differences in alpha diversity (using both Chao1 and Shannon indexes, p -value >0.05), despite an increasing trend in taxon abundance was found when comparing MK to DP variety (Fig. 6). Moreover, taxon abundance increased from CTRL to Rh treatment, with PG Rh showing intermediate values, except for MK Rh that showed the lowest taxa abundance. No differences were highlighted considering the beta diversity (Fig. S4a). Variety core microbiome showed some differences in taxon associations. Specifically, soil of variety DP was characterized by a high presence of Gaiellales, while MK one by the presence of Rubrobacterales and Frankiales. Taxon abundance at order level (Fig. 6) showed that most of the taxa had a similar trend in both varieties and among the different treatments. Despite that, some orders were more abundant in soil of DP compared to MK one and vice versa. In particular, soil of DP showed a higher abundance of Gaiellales, Burkholderiales, Thermomicrobiales, Tistrellales, Propionibacterales, Sphingomonadales and Streptomycetales. On the other hand, soil of MK showed a higher abundance of Rubrobacterales, Rhizobiales, Frankiales and Solirubrobacterales. Abundance analysis between the different treatments and the associated bacterial genera highlighted the presence of some representative taxa (Fig. 7a). *Bacillus* and *Solirubrobacter* represented the main taxa in all DP soil samples and in MK CTRL, while *Domibacillus* was uniquely associated with MK Rh one. Moreover, *Rubrobacter* was associated to all MK soil samples and DP Rh and *Solirubrobacter* to DP CTRL. The highest abundance of *Bacillus* was detected in DP PG Rh and MK CTRL samples, while the highest abundance of *Rubrobacter* in MK PG Rh ones.

The core microbiome of bacterial community of 2022 was represented by orders Gaiellales, Solirubrobacterales, Rubrobacterales, Bacillales and Tistrellales. Samples showed variation in alpha diversity considering the “inoculation” factor only considering Chao1 index (p -value 0.024). The interaction between “inoculation” and “variety” factors resulted as significant using both indexes (p -value 0.03 and p -value 0.018 for Chao1 and Shannon indexes, respectively, Fig. 6), while no differences were highlighted in beta diversity (Fig. S4b). Not-inoculated samples (CTRL) showed an overall lower associated-taxa diversity compared to the others. The highest taxa diversity was recorded in MK soil under PG Rh treatment. It is interesting to notice that, in DP soil, taxa diversity increased progressively from CTRL to Rh treatment, with PG Rh showing intermediate values. The core microbiome of DP soil samples showed specific orders associated to DP variety i.e., Propionibacterales, Micrococcales and Rhizobiales. On the other hand, the core microbiome of MK soil samples showed as uniquely associated taxa the orders Frankiales, Microtrichales, Nitrososphaerales and Vicinamicrobiales. Soil samples of MK variety showed a higher diversity among and inside treatments. Taxon abundance visualization (Fig. 6) revealed that, in DP variety soil, orders Gaiellales, Rubrobacterales, Micrococcales, Gemmatimonadales, Propionibacterales and Tistrellales were more abundant than in MK one, in which, on the contrary, orders Vicinamicrobiales, Frankiales, Microtrichales, Thermomicrobiales, Nitrososphaerales and Azospirillales were more abundant. Abundance analysis performed between the different treatments and the associated bacterial genera (Fig. 7b) showed that *Bacillus* and *Rubrobacter* were the most abundant taxa in the different treatments, in particular *Bacillus* higher abundance was detected in DP PG Rh and *Rubrobacter* in DP CTRL, DP Rh and MK PG Rh. Additionally, *Blastococcus* was associated with MK Rh samples.

The LEfSe analysis did not allow to detect any significant bacterial taxa associated with specific condition. On the other hand, results from ANCOM-BC allowed to identify 12 and 14 bacterial genera that showed significant positive and negative LFCs, respectively (Fig. 7c). Genera such as *Domibacillus*, *Allorhizobium*, *Noviherbaspirillum*, and *Rhodoplanes* showed a decrease in abundance under the inoculated treatments. Conversely, positive LFCs were detected in genera like *Roseisolibacter*, *Sphingomonas*, *Gaiella*, *Skermanella*, *Rubrobacter*, *Paenibacillus*, *Microbacterium*, *Roseomonas* and *Pseudomonas*, suggesting an increase in relative abundance of these taxa in response to the inoculation (Fig. 7c).

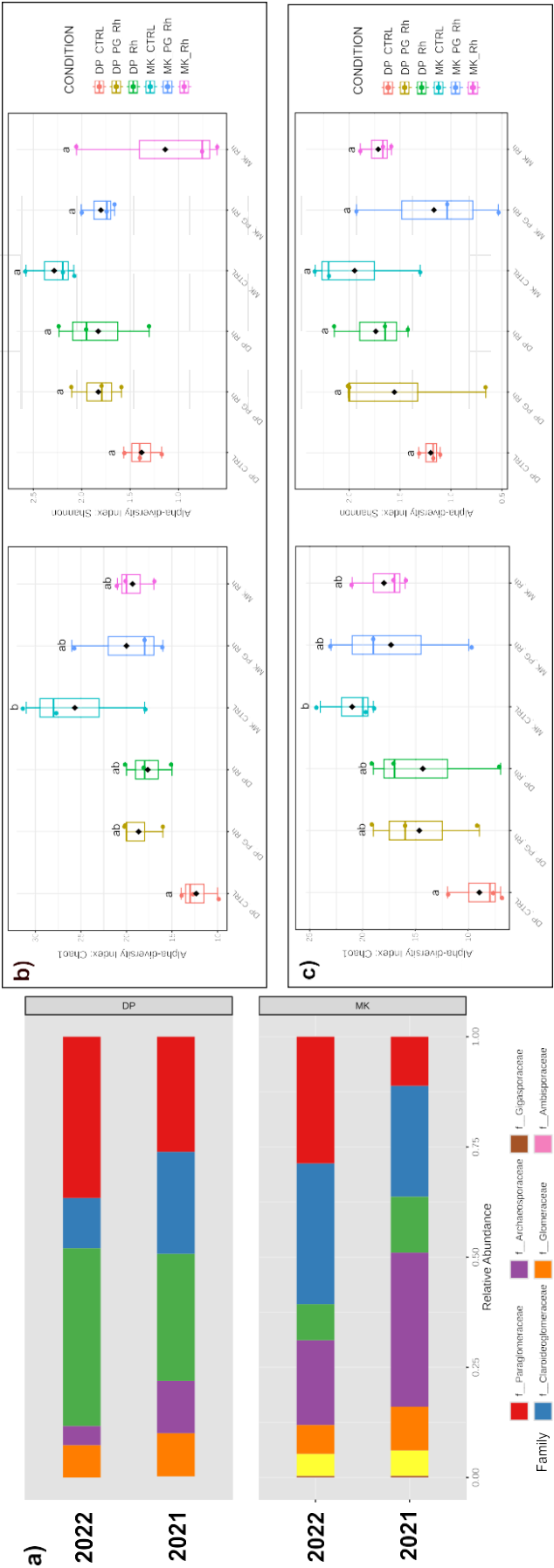


Fig. 4. AM fungal diversity and abundance in soil of DP and MK varieties subjected to the three inoculation conditions: a) AM families abundance in the two varieties in 2021 and 2022, b) alpha diversity of AM fungal community in 2021 and c) alpha diversity analysis (Chao1 and Shannon index on the left and on the right, respectively) was performed considering the interaction between the “variety” and the “inoculation” factors. DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with Rhizobium strain 25PS6, PG Rh: inoculated with Rhizobium 25PS6 and Erwinia sp. 12PS6 and sp. 13PS19 strains.

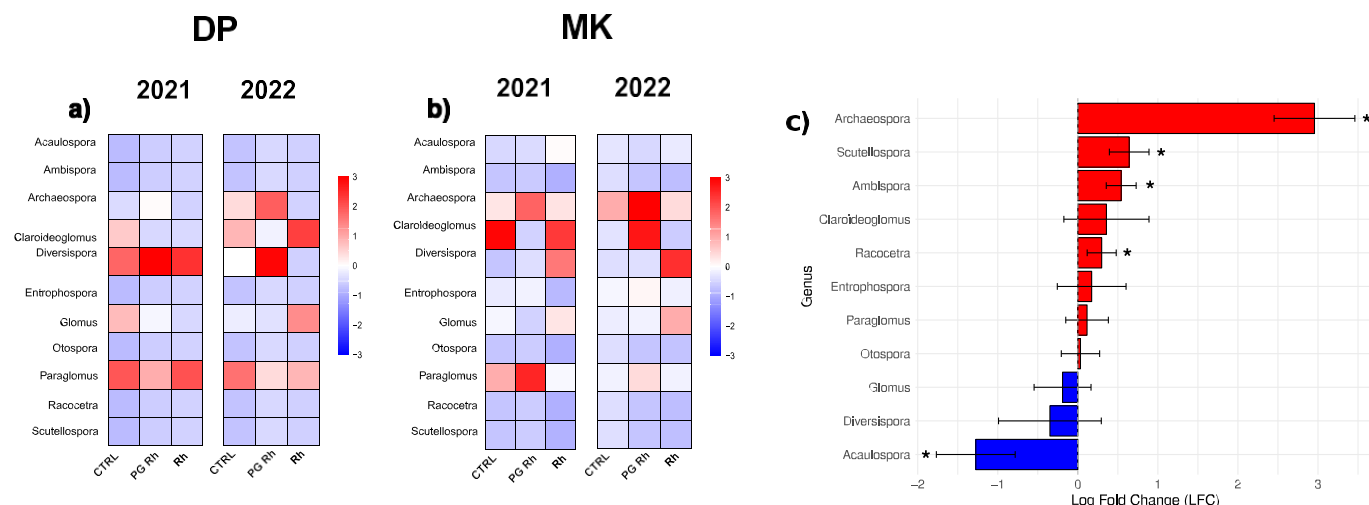


Fig. 5. Heatmaps of the most abundant AM fungal taxa and outcomes of ANCOM-BC at genus level. a) AM fungal taxa abundance in soil of DP variety subjected to the three inoculation conditions in 2021 and 2022; b) AM fungal taxa abundance in soil of MK variety subjected to the three inoculation conditions in 2021 and 2022; c) Differential abundance barplot from ANCOM-BC, where each bar represents a log fold-change (in natural log) value of inoculated treatments compared to not-inoculated ones and relative SE as error bars. Asterisks depict significant differences at p -value ≤ 0.05 . DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

3.5. Comparison between the two years and correlation between AM fungal and bacterial taxa

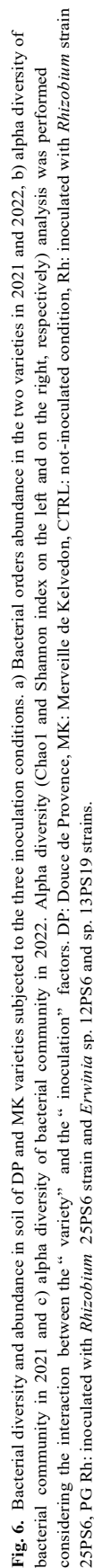
The AM fungal core microbiome was conserved between 2021 and 2022, with the exception of Archaeosporales that were absent in 2022 core. Comparison of AM fungal abundance performed between the two years highlighted that all the most abundant families were conserved (Fig. 4). In particular, DP variety soil showed a higher abundance of Diversisporaceae and Paraglomeraceae, while in MK soil, families Claroideoglomeraceae, Acaulosporaceae and Archaeosporaceae were the most abundant ones. Abundance analysis highlighted that *Claroideoglomus* genus was always associated to MK variety soil, despite its abundance varied among the different treatments of this variety (Fig. 5). Bacterial core microbiome showed that Solirubrobacterales and Bacillales were conserved between the two years, while the abundance of other taxa varied. In particular, in both years DP variety soil showed a higher abundance of Gaiellales, Propionibacterales and Tistrellales (Fig. 6). On the other hand, MK variety soil was characterized by the associated presence of Frankiales (Fig. 6). Abundance analysis revealed no conserved genera associated to the different treatments in the two years (Fig. 7). Overall, most of the associated taxa did not show variation in relation to the “variety”, the “inoculation” and the “year” factors.

Correlation analysis performed between AM fungal and bacterial community showed different correlated taxa in the two varieties soils (Fig. 8). Specifically, DP variety soil was characterized by a higher number of correlated taxa with respect to MK one. In DP variety soil, *Ambispora*, *Otospora* and *Racocetra* were correlated with *Microbacterium* and *Skermanella* in PG Rh treatment. Moreover, in PG Rh treatment and in Rh, *Archaeospora* was correlated with *Roseisolibacter*. *Entrophospora* was correlated with *Bryobacter*, *Domibacillus*, *Dongia*, *Massilia*, *Noviherbaspirillum* and *Tumebacillus* in CTRL. *Scutellospora* was correlated with *Gemmatimonas* in PG Rh and Rh treatments. In all treatments of DP variety *Glomus* was correlated with *Microbacterium*, while *Paraglomus* was correlated with *Rubrobacter* (Fig. 8a). Similarly to DP, in MK variety soil the correlations highlighted between *Glomus* and bacterial taxa were detected only in CTRL treatment. In particular, *Glomus* was correlated with *Gemmatimonas*, *Microbacterium* and *Noviherbaspirillum*. In all treatments, *Acaulospora* was correlated with *Bacillus* and *Scutellospora* with *Blastococcus*. Additionally, *Scutellospora* was also correlated with *Sphingomonas* in PG Rh and Rh treatments (Fig. 8b).

4. Discussion

4.1. Bacterial inocula positively affected plant biometric traits in both pea varieties

The two-year trial here conducted in open field conditions allowed to explore the response of two different pea varieties after rhizobia and PGPB inoculation, as well as the interaction between pea plants and soil microbiota, focusing on natural AM fungal and bacterial communities. Based on biometric parameters such as shoot length, shoot dry weight, number of pods, and pod weight, the estimated productivity of pea plants in 2021 were generally higher to that observed in 2022. A similar difference was recorded also considering the variety effect, with DP plants that were characterized by greater biometric parameters compared to MK one. However, for both varieties, the Rh and PG Rh inoculation had a positive impact on all the biometric parameters, especially on shoot dry weight, pod number and weight. Notably, results on roots showed that PG Rh inoculation was associated to a higher number of nodules, as well as to greater root dry weight, compared to the other inoculation conditions. In Tunisia, these results were similar to those reported by Hachana et al. (2021). The authors found that different pea varieties exhibited varying levels of adaptability to soil variations, with the long cycle variety showing the highest nodulation and adaptability to soil conditions (Hachana et al., 2021). A study of Abebaw (2024) performed in Ethiopia showed that rhizobia inoculation significantly enhanced various biometric parameters in *P. sativum*, including pod number, seed weight, and total seed yield. Another study showed that a *Pseudomonas* strain with PGP traits was able to boost pea growth under water deficit through physiological and metabolic changes, including an increased ABA content in shoots (Schilaci et al., 2024). Concerning leaf nitrate content, it has been previously demonstrated that PGPB inoculation is generally associated a greater root nitrate absorption (Aquino et al., 2021). Our results showed that leaf nitrate content was higher in inoculated samples compared to not-inoculated ones. This results can be explained by the fact that during the flowering stage of plant growth, plants require more nitrogen to support the development of flowers (Yun et al., 2023) and different studies demonstrate that PGPB are able to boost root N-absorption (Di Benedetto et al., 2017). As the plant transitions from the flowering stage to the pod formation stage, the nitrate concentration typically decreases. This reduction occurs because the plant shifts its focus from vegetative



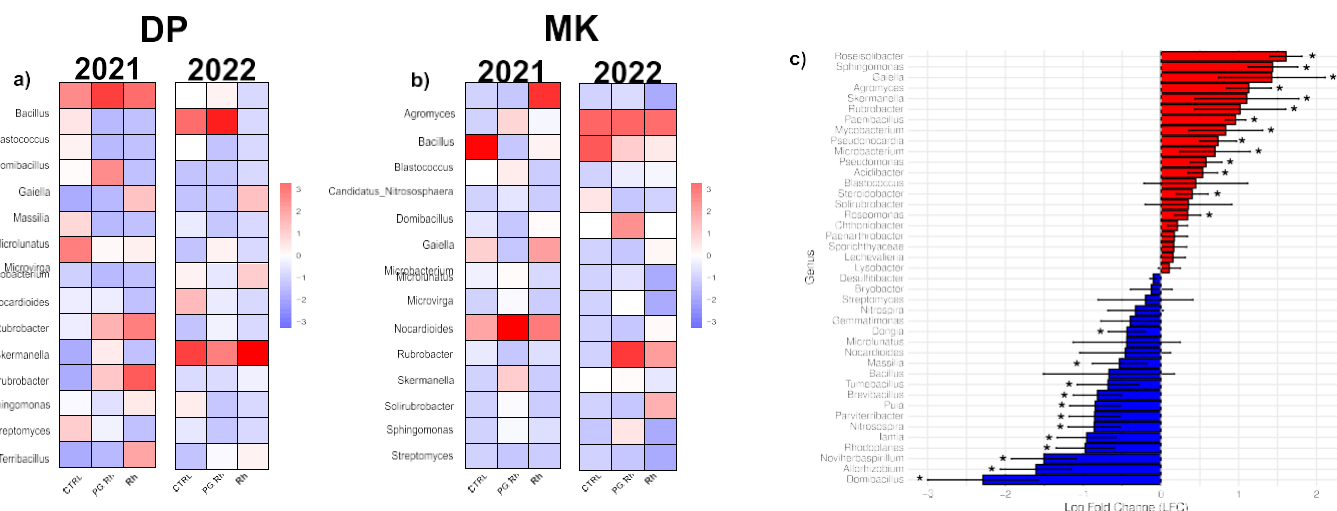


Fig. 7. Heatmaps of the most abundant bacterial taxa outcomes of ANCOM-BC at genus level. a) Bacterial genera abundance in soil of DP variety subjected to the three inoculation conditions in 2021 and 2022; b) Bacterial genera abundance in soil of MK variety subjected to the three inoculation conditions in 2021 and 2022; c) Differential abundance barplot from ANCOM-BC, where each bar represents a log fold-change (in natural log) value of inoculated treatments compared to not-inoculated ones and relative SE as error bars. Asterisks depict significant differences at p -value ≤ 0.05 . DP: Douce de Provence, MK: Merveille de Kelvedon, CTRL: not-inoculated condition, Rh: inoculated with *Rhizobium* strain 25PS6, PG Rh: inoculated with *Rhizobium* 25PS6 strain and *Erwinia* sp. 12PS6 and sp. 13PS19 strains.

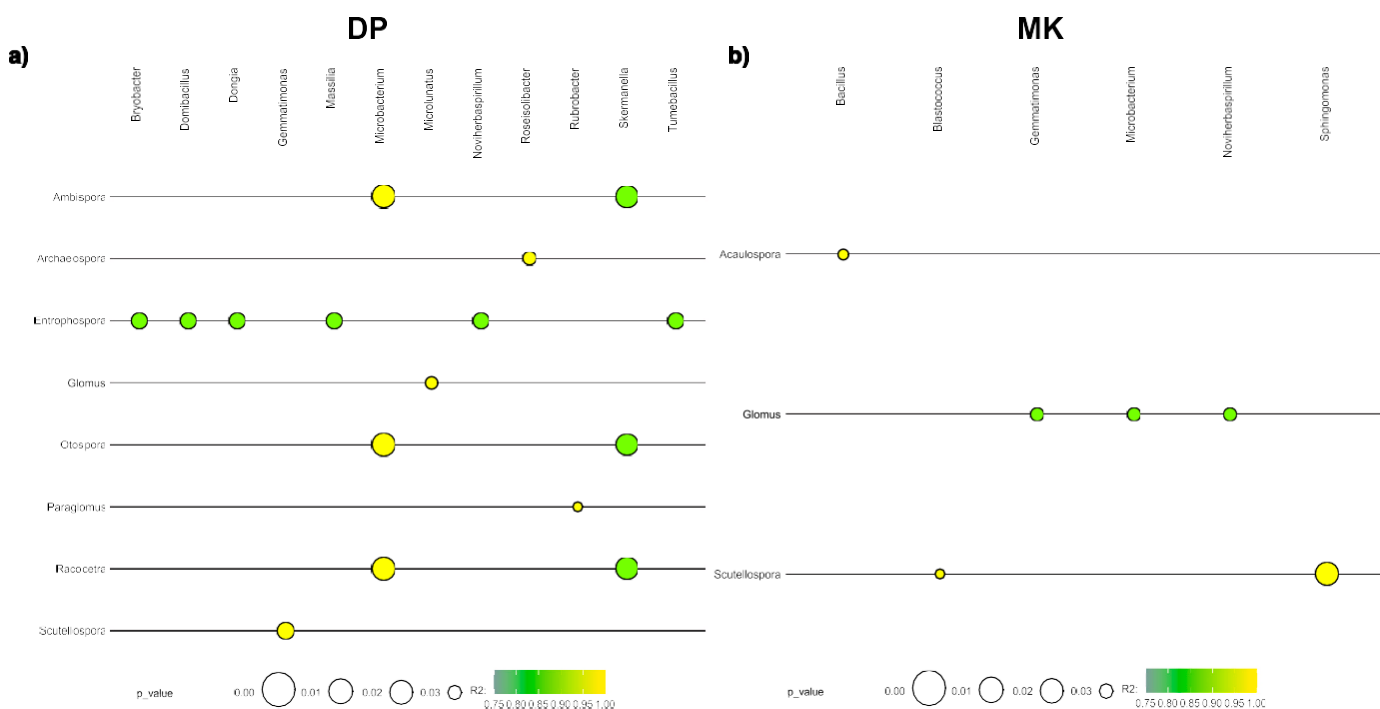


Fig. 8. Correlation analysis between AM fungal and bacterial communities in soils of the two varieties. a) Correlation analysis (Pearson correlation) between AM fungal and bacterial communities in soil of DP variety and b) correlation analysis (Pearson correlation) between AM fungal and bacterial communities in soil of MK variety. DP: Douce de Provence, MK: Merveille de Kelvedon. R and p values are reported.

growth to reproductive development. During the pod stage, the plant re-allocates its resources to the production and maturation of pods and seeds. As a result, the demand for nitrogen decreases, leading to lower nitrate levels in the plant tissues (Rahmat et al., 2023). Overall, PG Rh treatment, considering all the analyzed biometric parameters, in which the *Erwinia* strains have been used, resulted in improved plant biometric parameters compared to both control and Rh one, suggesting a beneficial role in promoting pea plants growth. *Erwinia* belongs to Enterobacterales (Adeolu et al., 2016), that includes several PGPB (Ramakrishnan et al., 2023) and the used strains have been selected because of their PGP

activity (Ilahi et al., 2024).

4.3 Soil AM fungal and bacterial alpha diversity increased in presence of the bacterial inoculation

In both years, in PG Rh inoculated parcels, higher AM fungal and bacterial diversity was detected with respect to control ones, suggesting a role of the inocula in improving microbiome diversity and abundance

in soil. Most of the AMF identified in this study have been previously reported in arid and semi-arid environments, and included *Glomus* sp., *Scutellospora* sp., *Ambiospora* sp., *Diversispora* sp. and *Paraglomus* sp., with *Glomus* sp. as the most distributed genus across the different environments (Torrecillas et al., 2012; M'saouar et al., 2019; Gritli et al., 2020; Guo et al., 2021; Ibou et al., 2021; Ashwin et al., 2023). Notably, in our study, the order Glomerales represented the AMF core identified for both varieties, as previously documented in Tunisian soils (Gritli et al., 2020). Additionally, variety-dependent AM fungal associations were identified and, specifically, in soil of MK variety a higher abundance of Archaeosporales, Claroidoglomerales and Acaulosporales was observed, while in DP variety the presence of Diversisporales and Paraglomerales was recorded. The LEfSe analysis showed that Acaulosporaceae, Gigasporaceae and Archaeosporaceae were more abundant in MK variety. These families include taxa previously highlighted as commonly found in Tunisian environments e.g., *Scutellospora* (Gritli et al., 2023). Additionally, *Diversispora* and *Claroidoglossum* genera have been previously reported to be abundant in field of pea plants, and *Diversispora* has been suggested as a key group of AMF recruited by pea (Lee et al., 2023), as also observed for DP variety in our experiment. Different studies highlighted that AM fungal diversity can be also associated to P levels in the soil (Chu et al., 2013; Baltruschat et al., 2019; Xiao et al., 2019). A recent meta-analysis on global distribution of AMF has been suggested that limited P availability leads to increased AM fungal diversity (Ma et al., 2023). In our study, the soil showed an average level of P (89.56 µg/g), and this could explain the detected diversity in AM fungal communities, as previously observed (Chu et al., 2013; Xiao et al., 2019). Thus, considering the increase in AM fungal diversity triggered by Rh and PG Rh inoculation, it cannot be excluded that the identified improvement in pea biometric parameters may be due to both bacterial inocula and specific AM fungal associations established by each variety.

Concerning the bacterial communities, in rhizospheric soil of MK variety a higher presence of the order Frankiales was found, while in DP one a higher abundance of Gaiellales and Propionibacterales was recorded. It is noteworthy that Frankiales, Gaiellales, and Propionibacterales are members of the Actinobacteria phylum (Dworkin et al., 2006; Albuquerque and da Costa, 2014; Narayanasamy et al., 2020), a well-known group of bacteria that can be found in the mycorrhizosphere (Agnolucci et al., 2019; Ujvári et al., 2021) and that can act as PGPB (Caddell et al., 2020). Actinobacteria is reported as a group of bacteria that can improve plant fitness through several mechanisms, including inorganic soil phosphorus solubilization, nitrogen-fixing ability, as well as production of ACC-deaminase, siderophores, phytohormones and enzymes that may regulate plant growth (Mitra et al., 2022). Among Actinobacteria, the order Frankiales harbors taxa known to be able to establish symbiotic relationships with plant roots and also to live in soils as saprotroph (Mantelin and Touraine, 2004). They are considered the only Actinobacteria taxa that have N₂ fixing ability (Bouizgarne and Ait Ben Aouamar, 2014) and might show drought-resistant characteristics (Gupta et al., 2020). Interestingly, these bacteria are able to establish symbiotic relationships with a group of plants named actinorhizal plants (Benson and Dawson, 2007), which comprise non-leguminous plants belonging to eight angiosperm families and 24 genera (Narayanasamy et al., 2020). Considering that pea is a leguminous crop, the presence of Frankiales in soil of MK variety may, therefore, be explained by the presence of specific AMF rather than on the relationship with pea plants.

4.2. Different putative PGPB were associated with specific AM fungal groups

In our study, different correlations among AMF and bacteria were highlighted in both rhizospheric soils of DP and MK varieties. Specifically, in soil of DP variety *Microbacterium* and *Skermanella* were correlated with the AMF *Otospora* and *Racocetra*, while *Roseisolibacter* was found to be correlated with *Archaeospora*. The genus *Microbacterium* included strains considered as PGPB able to enhance shoot fresh weight in leguminous

plants such as *Trifolium pratense* (Martínez-Hidalgo and Hirsch, 2017). Its PGP activity relies on different mechanisms, including ACC deaminase and IAA production, plant ethylene reduction and the production of antifungal metabolites able to contrast plant pathogens (Chieb and Gachomo, 2023; Tsavkelova et al., 2024). This genus has also been observed as part of the endophytic community in pea seeds, along with *Bacillus*, *Sphingomonas*, and *Erwinia* (Chartrel et al., 2021). *Skermanella* genus includes mycorrhizal helper diazotrophic bacteria able to perform biological N-fixation (Nasuelli et al., 2023). *Skermanella* can act as a mycorrhizal rhizosphere indicator and can perform as PGPB via N-fixation and production of alkaline phosphatase (Chen et al., 2023). This genus has previously been reported as correlated with the presence of *Gaiella*, and this co-occurrence has been suggested as a driver of the improvement of the N-uptake in wheat plants grown in N-rich conditions (Chen et al., 2023). Notably, in our study the order Gaiellales, which includes *Gaiella* genus, was more abundant in soil of DP variety for which the correlation among *Skermanella* and the different AM fungal genera has been identified. *Roseisolibacter* belongs to Gemmatimonadaceae family (Zhang et al., 2003; Pascual et al., 2018) that includes P-solubilizers and bacteria able to induce plant hormone production (You et al., 2024).

On the other hand, in rhizospheric soil of MK variety, the AMF *Scutellospora* and *Glomus* were found to be correlated with *Sphingomonas* and *Gemmatimonas*, respectively. *Sphingomonas* includes diazotrophic bacteria (Nasuelli et al., 2023). This genus has been reported to be able to produce IAA (Chieb and Gachomo, 2023) and to improve shoot length and dry weight in soybean (Asaf et al., 2017). Moreover, its abundance seems to increase in *Lotus japonicus* rhizosphere in presence of AMF including *Glomus mosseae*, *Rhizophagus intraradices*, and *G. versiforme* (Xu et al., 2023). The genus *Gemmatimonas* has been previously observed as enriched in *L. japonicus* rhizosphere under AM fungal inoculation (Xu et al., 2023). In maize, it have been observed a positive correlation between abundance of *Acaulospora tuberculata* and *Brevibacillus* sp. (Yuttavanchakul et al., 2018). Additionally, a study on cacao plants inoculated with AM fungal-based inocula containing *Scutellospora* sp., demonstrated that *Bacillus* sp. was the most dominant genera in the rhizosphere of inoculated plants (Cortes et al., 2021). In our study, a positive correlation has been observed between *Acaulospora* sp. and *Bacillus* sp. (Fig. 8). Based on these results, it may be hypothesized that this particular AM fungal genus may be related to the presence of Bacillales members.

Differential abundance analysis performed with ANCOMBC method showed that some bacterial genus were associated with inoculated treatments. Several of the genera showing differential abundance matched the ones found in correlation analysis in DP and MK variety soils. Particularly, the increase in relative abundance of AMF taxa *Archaeospora*, *Racocetra* and *Scutellospora*, along with the increase of *Roseisolibacter*, *Gaiella*, *Skermanella*, *Sphingomonas* in inoculated treatments aligned with the correlation analysis, suggesting that bacterial inoculation promote the presence of different AMF taxa and several associated bacterial genera. Other bacteria genera showing a great abundance in inoculated treatments, such as *Rubrobacter*, *Paenibacillus*, *Pseudomonas* and *Roseomonas*, include members reported as PGPB and N₂-fixing bacteria living in semi-arid soils (Pickett et al., 2019; Dias et al., 2024; Schillaci et al., 2024). Thus, it could be hypothesized that the applied bacterial inoculation may promote the AMF diversity in soil as well as may favor the presence of putative PGPB. However, our work focused on rhizospheric soil samples and future researches could benefit from the inclusion of root samples to provide further insights on root-rhizosphere system. Root microbiome is in fact considered as a part of the tripartite interactions involving plant roots, rhizosphere and bulk soil (Gregory, 2006).

5. Conclusions

In conclusion, our study demonstrated the positive effect of inoculation with rhizobia and the *Erwinia* strains on two pea cultivars in field conditions. Inoculation with these bacteria positively impacted all biometric parameters, and DP variety showed greater values compared to MK. High bacterial and AM fungal diversity in soil of PG Rh inoculated conditions was observed, suggesting that this bacterial consortium enhances the rhizospheric soil microbial diversity and abundance. The analysis of the soil bacterial core microbiome revealed distinct associations for each pea variety and, particularly, some taxa belonging to Actinobacteria reported as PGPB were identified. This suggests that the specific bacterial communities, associated with each variety, may play a crucial role in promoting plant growth and productivity. Notably, it has been reported that AM fungal mycorrhizosphere hosts different bacterial taxa, including bacterial strains recognized for their PGP traits (Agnolucci et al., 2019). Correlations between several bacterial and AM fungal taxa, e.g., *Microbacterium* and *Skermanella* and the AM fungal taxa *Otospora* and *Racocetra* in the DP cultivar, as well as *Sphingomonas* and *Scutellospora* in MK cultivar, suggested a relevant role of the microbial communities of the mycorrhizosphere in improving both soil health and pea plant biometric parameters. Overall, our study highlights the potential of tailored inoculation approaches to improve pea plant growth and productivity by exploiting the synergistic effects of soil microbial interactions in the frame of sustainable agriculture practices.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.apsoil.2024.105757>.

CRedit authorship contribution statement

Houda Ilahi: Writing – original draft, Methodology, Investigation, Formal analysis. **Alice Calvo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Sana Dhane:** Methodology. **Mustapha Missbah El Idrissi:** Methodology. **Lahcen Ouahmane:** Methodology. **Mohamed Najib Alfeddy:** Methodology. **Thomas Reitz:** Writing – review & editing, Methodology, Formal analysis. **Fabiano Sillo:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Raffaella Balestrini:** Writing – review & editing, Supervision, Methodology, Investigation. **Bacem Mnasri:** Writing – review & editing, Methodology, Investigation, Conceptualization.

Funding

The work was partially funded by the PRIMA RESCHEDULE project (Italian MUR DD 1293/2021, German BMBF 01DH21019 and Tunisian PRIMA/TN/20/06) and by the Tunisian-Moroccan bilateral project (20/PRD03).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge the support of Beatrix Schnabel from the Department of Soil Ecology (UFZ) for the sequencing analysis and of Saïf-Allah Chihoui from the Centre of Biotechnology of Borj-Cédria for the field work. All contributions made by members of the Centre of Biotechnology of Borj-Cédria were sincerely appreciated.

Data availability

Reads from metabarcoding of soil samples are available at NCBI SRA database under BioProject ID PRJNA1141918. All other data are included in the manuscript and its Supplementary materials.

References

- Abeba, B.G., 2024. Effect of Rhizobium bacteria inoculation rate on yield and yield components of field pea (*Pisum sativum* L.) at Awi zone. Ethiopia 81. <https://doi.org/10.15835/buasvmcn-agr.2023.0006> bulletin of university of agricultural sciences and veterinary medicine cluj-napoca. agricultura.
- Adeolu, M., Alnajjar, S., Naushad, S., Gupta, R.S., 2016. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales Ord. Nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. Nov., Pectobacteriaceae fam. Nov., Yersiniaceae fam. Nov., Hafniaceae fam. Nov., Morganellaceae fam. Nov., and Budviciaceae fam. Nov. Int. J. Syst. Evol. Microbiol. 66, 5575–5599. <https://doi.org/10.1099/ij-sem.0.001485>.
- Agnolucci, M., Palla, M., Cristani, C., Cavallo, N., Giovannetti, M., De Angelis, M., Gobetti, M., Minervini, F., 2019. Beneficial plant microorganisms affect the endophytic bacterial communities of durum wheat roots as detected by different molecular approaches. Front. Microbiol. 10, 2500. <https://doi.org/10.3389/fmicb.2019.02500>.
- Albuquerque, L., da Costa, M.S., 2014. The family Gaiellaceae. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.), The Prokaryotes. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-30138-4_394.
- Aquino, J.P.A., Antunes, J.E.L., Bonifacio, A., Rocha, S.M.B., Amorim, M.R., Alântara Neto, F., Araujo, A.S.F., 2021. Plant growth-promoting bacteria improve growth and nitrogen metabolism in maize and sorghum. Theor. Exp. Plant Physiol. 33, 249–260. <https://doi.org/10.1007/s40626-021-00209-x>.
- Arora, N.K., Mehraz, S., Balestrini, R. (Eds.), 2016. Bioformulations: for sustainable agriculture. Springer India, New Delhi. <https://doi.org/10.1007/978-81-322-2779-3>.
- Asaf, S., Khan, A.L., Khan, M.A., Imran, Q.M., Yun, B.-W., Lee, I.-J., 2017. Osmoprotective functions conferred to soybean plants via inoculation with *Sphingomonas* sp. LK11 and exogenous trehalose. Microbiol. Res. 205, 135–145. <https://doi.org/10.1016/j.micres.2017.08.009>.
- Ashwin, R., Bagyaraj, D.J., Raju, B.M., 2023. Dual inoculation with *Bradyrhizobium liaoningense* and *Ambispora leptoticha* improves drought stress tolerance and productivity in soybean cultivars, MAUS 2 and DSR 12. Biologia 78, 331–348. <https://doi.org/10.1007/s11756-022-01196-3>.
- Balestrini, R., Sillo, F., Boussageon, R., Wipf, D., Courty, P.E., 2024. The hidden side of interaction: microbes and roots get together to improve plant resilience. J. Plant Interact. 19, 232399. <https://doi.org/10.1080/17429145.2024.2323991>.
- Baltruschat, H., Santos, V.M., da Silva, D.K.A., Schellenberg, I., Deubel, A., Sieverding, E., Oehl, F., 2019. Unexpectedly high diversity of arbuscular mycorrhizal fungi in fertile Chernozem croplands in Central Europe. CATENA 182, 104135. <https://doi.org/10.1016/j.catena.2019.104135>.
- Barhoumi, S., Ellouzi, H., Krouma, A., 2023. Functional analysis of the genotypic differences in response of pea (*Pisum sativum* L.) to calcareous-induced iron deficiency. Phyton 92, 521–536. <https://doi.org/10.32604/phyton.2022.023555>.
- Benson, D.R., Dawson, J.O., 2007. Recent advances in the biogeography and genecology of symbiotic *Frankia* and its host plants. Physiol. Plant. 130, 318–330. <https://doi.org/10.1111/j.1399-3054.2007.00934.x>.
- Besser, H., Dhauadi, L., Hadji, R., Hamed, Y., Jemmal, H., 2021. Ecologic and economic perspectives for sustainable irrigated agriculture under arid climate conditions: an analysis based on environmental indicators for southern Tunisia. J. Afr. Earth Sci. 177, 104134. <https://doi.org/10.1016/j.jafrearsci.2021.104134>.
- Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo Rodriguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolk, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIVER, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Priesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez, Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. <https://doi.org/10.1038/s41587-019-0209-9>.
- Bouizgarne, B., Ait Ben Aouamar, A., 2014. Diversity of plant associated Actinobacteria. In: Maheshwari, D.K. (Ed.), Bacterial Diversity in Sustainable Agriculture. Springer International Publishing, Cham, pp. 41–99. https://doi.org/10.1007/978-3-319-05936-5_3.
- Caddell, D., Louie, K., Bowen, B., Sievert, J., Hollingsworth, J., Dahlberg, J., Purdom, E., Northen, T., Coleman-Derr, D., 2020. Drought shifts sorghum root metabolite and microbiome profiles and enriches the stress response factor picecolic acid. Phytobiomes J. 7, 449–463. <https://doi.org/10.1101/2020.11.08.373399>.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceed. Nat. Acad. Sci. 108, 4516–4522. <https://doi.org/10.1073/pnas.1000080107>.

- Chartrel, V., Dugat-Bony, E., Sarthou, A.-S., Huchette, S., Bonnarme, P., Irlinger, F., 2021. The microbial community associated with pea seeds (*Pisum sativum*) of different geographical origins. *Plant and Soil* 462, 405–427. doi: <https://doi.org/10.1007/s11104-021-04856-6>.
- Chen, M., Xu, J., Li, Z., Li, D., Wang, Q., Zhou, Y., Guo, W., Zhang, J., Zhao, B., 2023. Combined pot experiments and subsequent DNA-SIP incubations reveal a core microbiota involved in modulating crop nitrogen uptake derived from soil. *Appl. Soil Ecol.* 192, 105098. <https://doi.org/10.1016/j.apsoil.2023.105098>.
- Chieb, M., Gachomo, E.W., 2023. The role of plant growth promoting rhizobacteria in plant drought stress responses. *BMC Plant Biol.* 23, 407. <https://doi.org/10.1186/s12870-023-04403-8>.
- Chong, J., Liu, P., Zhou, G., Xia, J., 2020. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* 15, 3. <https://doi.org/10.1038/s41596-019-0264-1>.
- Chu, Q., Wang, X., Yang, Y., Chen, F., Zhang, F., Feng, G., 2013. Mycorrhizal responsiveness of maize (*Zea mays* L.) genotypes as related to releasing date and available P content in soil. *Mycorrhiza* 23, 497–505. <https://doi.org/10.1007/s00572-013-0492-0>.
- Cortes, A.D., Aggangan, N.S., Opuencia, R.B., 2021. Taxonomic microbiome profiling and abundance patterns in the cacao (*Theobroma cacao* L.) rhizosphere treated with arbuscular mycorrhizal fungi and bamboo biochar. *Philipp Agric Sci* 104, 19–33.
- Dalli, Y., Noureddine, Y., Abdelkader, B., 2020. Diversity of arbuscular mycorrhizal fungi associated with carob trees (*Ceratonia siliqua* L.) in western Algeria. *Plant Cell Biotechnol. Mol. Biol.* 21, 180–193.
- Dhariwal, A., Chong, J., Habib, S., King, I.L., Agellon, L.B., Xia, J., 2017. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acid Res.* 45, 180–188. <https://doi.org/10.1093/nar/gkx295>.
- Di Benedetto, N.A., Corbo, M.R., Campaniello, D., Cataldi, M.P., Bevilacqua, A., Sinigaglia, M., Flagella, Z., 2017. The role of plant growth promoting bacteria in improving nitrogen use efficiency for sustainable crop production: a focus on wheat. *AIMS Microbiol.* 3, 413–434. <https://doi.org/10.3934/microbiol.2017.3.413>.
- Dias, M.C., Silva, S., Galhano, C., Lorenzo, P., 2024. Olive tree belowground microbiota: plant growth-promoting bacteria and fungi. *Plants* 13, 1848. <https://doi.org/10.3390/plants13131848>.
- dos Santos Sousa, W., Soratto, R.P., Peixoto, D.S., Campos, T.S., da Silva, M.B., Souza, A. G.V., Teixeira, I.R., Gitari, H.I., 2022. Effects of *Rhizobium* inoculum compared with mineral nitrogen fertilizer on nodulation and seed yield of common bean. A meta analysis. *Agron. Sustain. Dev.* 42, 52. <https://doi.org/10.1007/s13593-022-00784-6>.
- Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E., 2006. *The Prokaryotes*. Springer, New York, New York, NY. <https://doi.org/10.1007/0-387-30743-5>.
- Ejaz, S., Batool, S., Anjum, M.A., Naz, S., Qayyum, M.F., Naqqash, T., Shah, K.H., Ali, S., 2020. Effects of inoculation of root-associative *Azospirillum* and *Agrobacterium* strains on growth, yield and quality of pea (*Pisum sativum* L.) grown under different nitrogen and phosphorus regimes. *Sci. Hortic.* 270, 109401. <https://doi.org/10.1016/j.scienta.2020.109401>.
- El Hazzat, N., Artib, M., Touati, J., Chliye, M., Selmaoui, K., Ouazzani Touhami, A., Benkirane, R., Douira, A., 2018. Diversity of endomycorrhizal fungi in the rhizosphere of chickpea in Morocco. *Acta Phytopathol. Entomol. Hung.* 53, 181–193. <https://doi.org/10.1556/038.53.2018.011>.
- Estaki, M., Jiang, L., Bokulich, N.A., McDonald, D., González, A., Kosciolk, T., Martino, C., Zhu, Q., Birmingham, A., Vázquez-Baeza, Y., Dillon, M.R., Bolyen, E., Caporaso, J.G., Knight, R., 2020. QIIME 2 enables comprehensive end-to-end analysis of diverse microbiome data and comparative studies with publicly available data. *Curr. Protoc. Bioinformatics* 70, e100. <https://doi.org/10.1002/cpbi.100>.
- George, N.P., Ray, J.G., 2023. The inevitability of arbuscular mycorrhiza for sustainability in organic agriculture. A critical review. *Front. Sustain. Food Syst.* 7. <https://www.frontiersin.org/articles/10.3389/fsufs.2023.1124688>.
- Giovannini, L., Palla, M., Agnolucci, M., Avio, L., Sbrana, C., Turrini, A., Giovannetti, M., 2020. Arbuscular mycorrhizal fungi and associated microbiota as plant biostimulants: research strategies for the selection of the best performing inocula. *Agronomy* 10, 1. <https://doi.org/10.3390/agronomy10010106>.
- Gregory, P.J., 2006. Roots, rhizosphere and soil: the route to a better understanding of soil science? *Eur. J. Soil Sci.* 57, 2–12. <https://doi.org/10.1111/j.1365-2389.2005.00778.x>.
- Gritli, T., Ellouze, W., Chihaoui, S.-A., Barhoumi, F., Mhamdi, R., Mnasri, B., 2020. Genotypic and symbiotic diversity of native rhizobia nodulating red pea (*Lathyrus cicera* L.) in Tunisia. *Syst. Appl. Microbiol.* 43, 126049. <https://doi.org/10.1016/j.syapm.2019.126049>.
- Gritli, T., Ellouze, W., Wu, H., Taamali, W., Fu, W., Alfeddy, M.N., Ouahmane, L., Courty, P.E., Wipf, D., El Idrissi, M.M., Chen, B., Mnasri, B., 2023. Molecular characterization of arbuscular mycorrhizal communities associated with *Lathyrus cicera* L. grown in northern Tunisia soils. *Symbiosis* 90, 81–90. <https://doi.org/10.1007/s13199-023-00922-y>.
- Guo, X., Wang, Z., Zhang, J., Wang, P., Li, Y., Ji, B., 2021. Host-specific effects of arbuscular mycorrhizal fungi on two Caragana species in desert grassland. *J. Fungi* 7, 1077. <https://doi.org/10.3390/jof7121077>.
- Gupta, S.M., Kumar, K., Joshi, R.K., Gupta, S., Bala, M., 2020. Frankia: A promising N-fixing plant growth promoting rhizobacteria (PGPR) improved drought tolerance in crops at higher altitude. In: Goel, R., Soni, R., Suyal, D.C. (Eds.), *Microbiological Advancements for Higher Altitude Agro-Ecosystems & Sustainability*. Springer, Singapore, pp. 411–431. https://doi.org/10.1007/978-981-15-1902-4_20.
- Hachana, A., Hemissi, I., Souissi, A., L'Taief, B., Abdi, N., Bouraoui, M., Al-Qthanin, R., Arfaoui, H., Sifi, B., 2021. Patterns for pea-*Rhizobium* symbiosis efficiency response to pedological and varietal variations in Tunisia. *Rhizosphere* 17, 1–10. <https://doi.org/10.1016/j.rhisph.2020.100304>.
- Hakim, S., Naqqash, T., Nawaz, M.S., Laraib, I., Siddique, M.J., Zia, R., Mirza, M.S., Imran, A., 2021. Rhizosphere engineering with plant growth-promoting microorganisms for agriculture and ecological sustainability. *Front. Sustain. Food Syst.* 5. <https://www.frontiersin.org/articles/10.3389/fsufs.2021.617157>.
- Ibou, D., Fatou, N., Diedhiou, A., Krasova-Wade, T., Rego, D., Kandiora, N., Ambrosi, J.-P., Aboubacry, K., 2021. Diversity and spore density of arbuscular mycorrhizal fungi in the rhizosphere of cowpea (*Vigna unguiculata* L. Walp.) cultivated in different soils in Senegal. *J. Anim. Plant Sci.* 48, 8552–8565. <https://doi.org/10.35759/JAnmPLSci.v48-1.1>.
- Ilahi, H., Hsouna, J., Ellouze, W., Gritli, T., Chihaoui, S., Barhoumi, F., Najib Elfeddy, M., Bachkouel, S., Ouahmane, L., Tambong, J.T., Mnasri, B., 2021. Phylogenetic study of rhizobia nodulating pea (*Pisum sativum*) isolated from different geographic locations in Tunisia. *Syst. Appl. Microbiol.* 44, 126221. <https://doi.org/10.1016/j.syapm.2021.126221>.
- Ilahi, H., Zampieri, E., Sbrana, C., Brescia, F., Giovannini, L., Mahmoudi, R., Gohari, G., El Idrissi, M.M., Alfeddy, M.N., Schillaci, M., Ouahmane, L., Calvo, A., Sillo, F., Fotopoulos, V., Balestrini, R., Mnasri, B., 2024. Impact of two *Erwinia* sp. on the response of diverse *Pisum sativum* genotypes under salt stress. *Physiol. Mol. Biol. Plants* 30, 249–267. <https://doi.org/10.1007/s12298-024-01419-8>.
- Kasotia, A., Varma, A., Tuteja, N., Choudhary, D.K., 2016. Microbial-mediated amelioration of plants under abiotic stress: An emphasis on arid and semiarid climate. In: Choudhary, Devendra K., Varma, A., Tuteja, N. (Eds.), *Plant-Microbe Interaction: An Approach to Sustainable Agriculture*. Springer, Singapore, pp. 155–163. https://doi.org/10.1007/978-981-10-2854-0_7.
- Lee, A., Neuburger, P., Omokanye, A., Hernandez-Ramirez, G., Kim, K., Gorzelak, M.A., 2023. Arbuscular mycorrhizal fungi in oat-pea intercropping. *Sci. Rep.* 13, 390. <https://doi.org/10.1038/s41598-022-22743-7>.
- Lin, H., Peddada, S.D., 2020. Analysis of compositions of microbiomes with bias correction. *Nat. Commun.* 11, 3514. <https://doi.org/10.1038/s41467-020-17041-7>.
- Lindström, K., Mousavi, S.A., 2020. Effectiveness of nitrogen fixation in *rhizobia*. *J. Microbiol. Biotechnol.* 13, 1314–1335. <https://doi.org/10.1111/1751-7915.13517>.
- Lu, Z.X., He, J.F., Zhang, Y.C., Bing, D.J., 2019. Composition, physicochemical properties of pea protein and its application in functional foods. *Crit. Rev. Food Sci. Nutr.* 60, 2593–2605. <https://doi.org/10.1080/10408398.2019.1651248>.
- Ma, X., Xu, X., Geng, Q., Luo, Y., Ju, C., Li, Q., Zhou, Y., 2023. Global arbuscular mycorrhizal fungal diversity and abundance decreases with soil available phosphorus. *Glob. Ecol. Biogeogr.* 32, 1423–1434. <https://doi.org/10.1111/geb.13704>.
- Mahmoudi, N., Mahdhi, M., Abdaiem, R., Bessadok, K., Mars, M., 2019. Arbuscular mycorrhizal colonization of selected herbaceous plants under arid protected area in Tunisia. *Soil Sci. Plant Nutr.* 65, 114–121. <https://doi.org/10.1080/00380768.2019.1579045>.
- Mahmoud, G.A.E., Abeer, A.H., Mostafa, H.H., Abdel Monsef, O., 2023. Responses of pea (*Pisum sativum* L.) to single and consortium bio-fertilizers in clay and newly reclaimed soils. *Plants* 12 (23), 3931. <https://doi.org/10.3390/plants12233931>.
- Mahmoudi, N., Dias, T., Mahdhi, M., Cruz, C., Mars, M., Caeiro, M.F., 2020. Does arbuscular mycorrhiza determine soil microbial functionality in nutrient-limited Mediterranean arid ecosystems? Diversity 12, 6. <https://doi.org/10.3390/d12060234>.
- Mantelin, S., Touraine, B., 2004. Plant growth-promoting bacteria and nitrate availability: impacts on root development and nitrate uptake. *J. Exp. Bot.* 55, 27–34. <https://doi.org/10.1093/jxb/erh010>.
- Mao, H., Whalen, J.K., Zhang, Z., Sheng, X., Hu, G., Chen, B., Ma, M., 2024. Arbuscular mycorrhizal fungi associated with alpine meadow multifunctionality in a warmer climate with variable precipitation. *Soil Biol. Biochem.* 198, 109555. <https://doi.org/10.1016/j.soilbio.2024.109555>.
- Martinez-Hidalgo, P., Hirsch, A.M., 2017. The nodule microbiome: N₂-fixing rhizobia do not live alone. *Phytophiles* J. 1, 70–82. <https://doi.org/10.1094/PBIOMES-12-16-0019-RVW>.
- Maxted, N., Bennett, S.J., 2001. Legume diversity in the Mediterranean region. In: Maxted, S. J. Bennett (a c. Di), *Plant genetic resources of legumes in the Mediterranean*, 51–75. Springer Netherlands. doi:https://doi.org/10.1007/978-94-015-9823-1_3.
- Missbah El Idrissi, M., Lamin, H., Bouhnik, O., Lamrabet, M., Alami, S., Jabrone, Y., Benis, M., Bedmar, E.J., Abdelmoumen, H., 2020. Characterization of *Pisum sativum* and *Vicia faba* microsymbionts in Morocco and definition of symbiovar *viciae* in *Rhizobium acidisoli*. *Syst. Appl. Microbiol.* 43, 126084. <https://doi.org/10.1016/j.syapm.2020.126084>.
- Mitra, D., Mondal, R., Khoshru, B., Senapati, A., Radha, T., Mahakur, B., Uniyal, N., Myo, E.M., Boutaj, H., Guerra Sierra, B.E., Panneerselvam, P., Ganeshamurthy, A., Andjelkovic, S., Vasic, T., Teotia, A., Dutta, S., Mohapatra, P., 2022. Actinobacteria enhanced plant growth, nutrient acquisition, and crop protection: advances in soil, plant, and microbial multifactorial interactions. *Pedosphere* 32, 149–170. [https://doi.org/10.1016/S1002-0160\(21\)60042-5](https://doi.org/10.1016/S1002-0160(21)60042-5).
- Moll, J., Kellner, H., Leonhardt, S., Stengel, E., Dahl, A., Bäessler, C., Buscot, F., Hofrichter, M., Hoppe, B., 2018. Bacteria inhabiting deadwood of 13 tree species are heterogeneously distributed between sapwood and heartwood. *Environ. Microbiol.* 20, 3744–3756. <https://doi.org/10.1111/1462-2920.1437>.
- M'saouar, R., Bakkali, M., Laglaoui, A., Arakrak, A., 2019. Evaluation of the mycorrhizal potential of *Hedysarum flexuosum* L. in relation with the soil chemical characteristics in the northwest of Morocco. *Moroccan J. Biol.* 16, 13–18.
- Narayanansamy, M., Dhanasekaran, D., Thajuddin, N., 2020. *Frankia*, in: beneficial microbes in agro-ecology. Chapter 11 *Frankia*, editor(s): N. Amarean, M. Senthil Kumar, K. Annapurna, Krishna Kumar, A. Sankaranarayanan, beneficial microbes in agro-ecology, academic press, 185–211. doi:<https://doi.org/10.1016/B978-0-12-823414-3.00011-3>.
- Nasuelli, M., Novello, G., Gamalero, E., Massa, N., Gorrasi, S., Sudiro, C., Hochart, M., Altissimo, A., Vuolo, F., Bona, E., 2023. PGPR and/or AM fungi consortia affect tomato native rhizosphere microbiota. *Microorganisms* 11, 1891. <https://doi.org/10.3390/microorganisms11081891>.
- Nazir, N., Nisar, M., Ahmad, S., Wadood, S.F., Jan, T., Zahoor, M., Ahmad, M., Ullah, A., 2020. Characterization of phenolic compounds in two novel lines of *Pisum sativum* L. along with their in vitro antioxidant potential. *Environ. Sci. Pollut. Res. Int.* 27, 7639–7646. <https://doi.org/10.1007/s11356-019-07065-y>.
- Noreen, S., Yaseen, T., Iqbal, J., Abbasi, B.A., Farouk Elsadek, M., Eldin, S.M., Ijaz, S., Ali,

- I., 2023. Morphological and molecular characterizations of arbuscular mycorrhizal fungi and their influence on soil physicochemical properties and plant nutrition. ACS Omega 8, 32468–32482. <https://doi.org/10.1021/acsomega.3c02489>.
- Opik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J.M., Reier, U., Zobel, M., 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). New Phytol. 188, 223–241. <https://doi.org/10.1111/j.1469-8137.2010.03334.x>.
- Pasquali, J., Foessel, B.U., Geppert, A., Huber, K.J., Boedeker, C., Luckner, M., Wanner, G., Overmann, J., 2018. Roseolibacter Agri gen. Nov., sp. nov., a novel slow-growing member of the under-represented phylum Gemmatimonadetes. Int. J. Syst. Evol. Microbiol. 68, 1028–1036. <https://doi.org/10.1099/ijsem.0.002619>.
- Pasquini, D., Zampieri, E., Ioannou, A., Spanos, A., Sillo, F., Giovannini, L., Fotopoulos, V., Brunetti, C., Lumini, E., Balestrini, R., 2023. Impact of the arbuscular mycorrhizal fungal inoculation on growth and biochemical parameters in *Rosmarinus officinalis* and *Lavandula angustifolia*. Symbiosis 91, 107–117. <https://doi.org/10.1007/s13199-023-00946-4>.
- Philippot, L., Chenu, C., Kappler, A., Rillig, M.C., Fierer, N., 2023. The interplay between microbial communities and soil properties. Nat. Rev. Microbiol. 22, 226–239. <https://doi.org/10.1038/s41579-023-00980-5>.
- Pickett, B., Irvine, I.C., Bullock, E., Arogyaswamy, K., Aronson, E., 2019. Legacy effects of invasive grass impact soil microbes and native shrub growth. Invas. Plant Sci. Manag. 12, 22–35. <https://doi.org/10.1017/imp.2018.32>.
- Poole, P., Ramachandran, V., Terpolilli, J., 2018. *Rhizobia*: from saprophytes to endosymbionts. Nat. Rev. Microbiol. 16, 291–303. <https://doi.org/10.1038/nrmicro.2017.171>.
- Prada-Salcedo, L.D., Goldmann, K., Heintz-Buschart, A., Reitz, T., Wambsganss, J., Bauhus, J., Buscot, F., 2021. Fungal guilds and soil functionality respond to tree community traits rather than to tree diversity in European forests. Mol. Ecol. 30, 572–591. <https://doi.org/10.1111/mec.15749>.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, 590–596. <https://doi.org/10.1093/nar/gks1219>.
- Radhouane, L., 2018. Why don't adapt Tunisian agriculture to climate change? 1. Climate change and agriculture in Tunisia. Int. J. Sci. Environ. Technol. 7, 1495–1508. ISSN 2278–3687.
- Rahmat, Z., Sohail, M.N., Perrine-Walker, F., Kaiser, B.N., 2023. Balancing nitrate acquisition strategies in symbiotic legumes. Planta 258, 12. <https://doi.org/10.1007/s00425-023-04175-3>.
- Ramakrishnan, P., Ariyan, M., Rangasamy, A., Rajasekaran, R., Ramasamy, K., Murugaiyan, S., Janahiraman, V., 2023. Draft genome sequence of *Enterobacter cloacae* S23 a plant growth-promoting passenger endophytic bacterium isolated from groundnut nodule possesses stress tolerance traits. Curr. Genomics 24, 36–47. <https://doi.org/10.2174/1389202924666230403123208>.
- Rubiales, D., Fondevilla, S., Chen, W., Gentzbitel, L., Higgins, T.J.V., Castillejo, M.A., Singh, K.B., Rispail, N., 2014. Achievements and challenges in legume breeding for pest and disease resistance. Crit. Rev. Plant Sci. 34, 195–236. <https://doi.org/10.1080/07352689.2014.898445>.
- Rubiales, D., Osuna-Caballero, S., González-Bernal, M.J., Cobos, M.J., Flores, F., 2021. Pea breeding lines adapted to autumn sowings in broomrape prone mediterranean environments. Agronomy 11, 769. <https://doi.org/10.3390/agronomy11040769>.
- Scheublin, T.R., Sanders, I.R., Keel, C., van der Meer, J.R., 2010. Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi. Int. J. Syst. Evol. Microbiol. 4, 752–763. <https://doi.org/10.1038/ismej.2010.5>.
- Schillaci, M., Zampieri, E., Brunetti, C., Gori, A., Sillo, F., 2024. Root transcriptomic provides insights on molecular mechanisms involved in the tolerance to water deficit in *Pisum sativum* inoculated with *Pseudomonas* sp. Planta 259, 33. <https://doi.org/10.1007/s00425-023-04310-0>.
- Singh, V.K., Singh, A.K., Singh, P.P., Kumar, A., 2018. Interaction of plant growth promoting bacteria with tomato under abiotic stress: a review. Agric. Ecosyst. Environ. 267, 129–140. <https://doi.org/10.1016/j.agee.2018.08.020>.
- Stewart, A.J., Chapman, W., Jenkins, G.I., Graham, I., Martin, T., Crozier, A., 2001. The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. Plant Cell Environ. 24, 1189–1197. <https://doi.org/10.1046/j.1365-3040.2001.00768.x>.
- Torreillas, E., Alguacil, M.M., Roldán, A., 2012. Host preferences of arbuscular mycorrhizal fungi colonizing annual herbaceous plant species in semiarid Mediterranean prairies. Appl. Environ. Microbiol. 78, 6180–6186. <https://doi.org/10.1128/AEM.01287-12>.
- Tozlu, E., Karagoz, K., Babagil, G., Dizikisa, T., Büyümesini, B., Bakterilerin, T., Fasulyenin, K., 2012. Effect of some plant growth promoting bacteria on yield, yield components of dry bean (*Phaseolus vulgaris* L. cv. Aras 98). J. Fac. Agric. 43, 101–106.
- Trabelsi, D., Mhamdi, R., 2013. Microbial inoculants and their impact on soil microbial communities: a review. Biomed. Res. Int. 863240, 11. <https://doi.org/10.1155/2013/863240>.
- Trivedi, P., Leach, J.E., Tringe, S.G., Sa, T., Singh, B.K., 2020. Plant-microbiome interactions: from community assembly to plant health. Nat. Rev. Microbiol. 18, 607–621. <https://doi.org/10.1038/s41579-020-0412-1>.
- Tsavkelova, E.A., Volynchikova, E.A., Potekhina, N.V., Lavrov, K.V., Avtuh, A.N., 2024. Auxin production and plant growth promotion by *Microbacterium alboburpureum* sp. nov. from the rhizosphere of leafless *Chiloschista parishii* Seidenf. Orchid. Front. Plant Sci. 15. <https://doi.org/10.3389/fpls.2024.1360828>.
- Ujvári, G., Turrini, A., Avio, L., Agnolucci, M., 2021. Possible role of arbuscular mycorrhizal fungi and associated bacteria in the recruitment of endophytic bacterial communities by plant roots. Mycorrhiza 31, 527–544. <https://doi.org/10.1007/s00572-021-01040-7>.
- Vincent, J., 1970. A Manual for the Practical Study of the Root-Nodule bacteria: IBP Handbk 15 Oxford and Edinburgh: Blackwell Scientific Publications; 1970.
- Wahdan, S.F.M., Reitz, T., Heintz-Buschart, A., Schädler, M., Roscher, C., Breitreutz, C., Schnabel, B., Purahong, W., Buscot, F., 2021. Organic agricultural practice enhances arbuscular mycorrhizal symbiosis in correspondence to soil warming and altered precipitation patterns. Environ. Microbiol. 23, 6163–6176. <https://doi.org/10.1111/1462-2920.15492>.
- Williams, A., Sinanaj, B., Hoysted, G.A., 2024. Plant-microbe interactions through a lens: tales from the mycorrhizosphere. Ann. Bot. 133, 399–412. <https://doi.org/10.1093/aob/mcad191>.
- Xiao, D., Che, R., Liu, X., Tan, Y., Yang, R., Zhang, W., He, X., Xu, Z., Wang, K., 2019. Arbuscular mycorrhizal fungi abundance was sensitive to nitrogen addition but diversity was sensitive to phosphorus addition in karst ecosystems. Biol. Fertil. Soils 55, 457–469. <https://doi.org/10.1007/s00374-019-01362-x>.
- Xu, Y., Chen, Z., Li, X., Tan, J., Liu, F., Wu, J., 2023. The mechanism of promoting rhizosphere nutrient turnover for arbuscular mycorrhizal fungi attributes to recruited functional bacterial assembly. Mol. Ecol. 32, 2335–2350. <https://doi.org/10.1111/mec.16880>.
- Yadav, A.N., 2020. Plant microbiomes for sustainable agriculture: Current research and future challenges. In: Yadav, A.N., Singh, J., Rastegari, A.A., Yadav, N. (Eds.), Plant Microbiomes for Sustainable Agriculture. Springer International Publishing, Cham, pp. 475–482. https://doi.org/10.1007/978-3-030-38453-1_16.
- You, Y., Wang, L., Liu, X., Wang, X., Jiang, L., Ding, C., Wang, W., Zhang, D., Zhao, X., 2024. Interspecific plant interaction structures the microbiomes of poplar-soil interface to alter nutrient cycling and utilization. Microbiol. Spectr. 12. <https://doi.org/10.1128/spectrum.03368-23>.
- Young, C., Soto, D., Bahri, T., Brown, D.W., 2012. Building resilience for adaptation to climate change in the fisheries and aquaculture sector, proceedings of a joint FAO/ OECD Workshop 23–24 April 2012, ISBN 978–92–5–107373–5, 346.
- Yun, J., Wang, C., Zhang, F., Chen, L., Sun, Z., Cai, Y., Luo, Y., Liao, J., Wang, Y., Cha, Y., Zhang, X., Ren, Y., Wu, J., Hasegawa, P.M., Tian, C., Su, H., Ferguson, B.J., Gresshoff, P.M., Hou, W., Han, T., Li, X., 2023. A nitrogen fixing symbiosis-specific pathway required for legume flowering. Sci. Adv. 9. <https://doi.org/10.1126/sciadv.ade1150>.
- Yuttavanichakul, W., Teamtisong, K., Teaumroong, N., Boonkerd, N., Tittabutr, P., 2018. *Brevibacillus* sp. promotes maize root colonization by *Acaulospora tuberculata* and the alteration of associated plant protein responses. J. Plant Interact. 13, 543–554. <https://doi.org/10.1080/17429145.2018.1547844>.
- Zhang, H., Sekiguchi, Y., Hanada, S., Hugenholtz, P., Kim, H., Kamagata, Y., Nakamura, K., 2003. *Gemmatimonas aurantiaca* gen. Nov., sp. nov., a gram-negative, aerobic, polyphosphate-accumulating micro-organism, the first cultured representative of the new bacterial phylum Gemmatimonadetes phyl. Nov. Int. J. Syst. Evol. Microbiol. 53, 1155–1163. <https://doi.org/10.1099/ijms.0.02520-0>.

Chapter III

This chapter is dedicated to a second work in collaboration with Prof. Bacem Mnasri's research group. In particular, I collaborated with the PhD student Waad Bousselmi during her stage at CNR-IPSP. The work has been published in Plant and Soil in 2025.

A two-years field trial was set up in Mateur, Tunisia. The experiment consisted in the evaluation of different types of agricultural systems of *Brassica napus*, including intercropping with *Hedysarum coronarium*, intercropping with *Medicago sativa* and *B. napus* monoculture, on the performance of *B. napus* plants and the effect of these three different cultivation systems on the rhizospheric and soil bacteria.

This study explored the importance of choosing the right legume to intercrop with canola to reduce the dependence on nitrogen-based chemical fertilizers. In particular, the two legumes were associated with different putative PGPB, strengthening the hypothesis that the variety-dependent recruitment of specific bacteria may be reflected also in canola growth improvement.

Legume choice matters: different effects on *Brassica napus* agronomic performance and root-associated bacterial communities in intercropping systems

Waad Bousselmi · Alice Calvo · Takoua Gritli · Mustapha Missbah El Idrissi · Thomas Reitz · Fabiano Sillo · Raffaella Balestrini · Bacem Mnasri

Received: 15 July 2025 / Accepted: 23 August 2025
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2025

Abstract

Background and Aims *Brassica napus* L., an oilseed crop, is one of the three major crop groups contributing to global food production. It is generally grown as a monoculture and is very demanding in terms of nitrogen inputs. To meet the nutritional needs of *B. napus* and to reduce the excessive use of chemical inputs, intercropping with legumes plays an important role in agricultural ecosystems. This study aims to assess how different intercropping systems with two

forage legumes (*Medicago sativa* L. and *Hedysarum coronarium* L.) affect the biometric parameters of *B. napus* and influence the associated bacterial communities in the rhizosphere and root compartments.

Methods A two-year field experiment was conducted to assess how *M. sativa* L. and *H. coronarium* L. intercropping influences *B. napus* biometric parameters and bacterial communities in both rhizosphere and root compartments, with particular emphasis on inter-annual variability and temporal dynamics of plant–microbe interactions.

Results Our results showed that the agronomic parameters varied among the different cultivation systems and between the two growing seasons (2022 and 2023). *H. coronarium* proved to be the best legume to

Responsible Editor: Guiyao Zhou.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11104-025-07855-z>.

W. Bousselmi
Faculty of Sciences of Bizerte, University of Carthage,
7021 Zarzouna, Tunisia

W. Bousselmi · T. Gritli · B. Mnasri (✉)
Laboratory of Legumes and Sustainable Agroecosystems, Centre of Biotechnology of BorjCedria, BP 901,
2050 Hammam-Lif, Tunisia
e-mail: mnbacemm@yahoo.com; bacem.mnasri@cbbc.rnrt.tn

A. Calvo · F. Sillo
Institute for Sustainable Plant Protection (IPSP), National Research Council of Italy, Turin, Italy

A. Calvo Department of Agriculture and Desertification Research Centre, University of Sassari, Sassari, Italy

M. M. E. Idrissi
Faculty of Sciences, Centre de Biotechnologies Végétale Et Microbienne, Biodiversité Et Environnement, Mohammed V University in Rabat, Rabat, Morocco

T. Reitz
Department of Soil Ecology, Helmholtz Centre for Environmental Research GmbH – UFZ, Halle, Germany

T. Reitz
German Centre for Integrative Biodiversity Research (iDiv), Halle-Jena-Leipzig, Leipzig, Germany

be used in intercropping with *B. napus*, particularly under drought conditions. Thus, this legume demonstrated high potential for supporting oilseed production when water availability is limited, highlighting its value for climate-resilient cropping systems. The intercropped legume species also shaped the bacterial communities in both the rhizosphere and *B. napus* roots, with Vicinamibacterales and Caulobacterales orders that increased in legume-canola intercropping systems.

Conclusion This study demonstrates that in intercropping with legumes, the promotion of *B. napus* growth depends on the legume species choice. Moreover, intercropping practice affects bacterial composition, particularly in *B. napus* roots, suggesting different microbial recruitment mediated by the presence of a specific legume.

Keywords *B. napus* · Intercropping systems · Legumes · Bacterial microbiota · Agroecosystems

Introduction

Climate change and the rapid global warming are increasing the incidence of drought, posing a serious challenge to sustainable agricultural production across the globe (Hassan et al. 2021; Kogan et al. 2019). Drought or scarcity of water is one of the main abiotic stresses that limits plant growth, development and productivity (El-Badri et al. 2021; Sehgal et al. 2019). The Mediterranean area is considered as a drought-vulnerable spot and the drought-related consequences have been felt more in the southern countries, including Tunisia. This country has experienced a high variability of rainfall regimes with prolonged drought events and a significant increase in temperature (Rahmani et al. 2020).

Brassica napus L., also known as canola, is an oilseed crop and one of three major crop groups

that contributes to the global food market production (Lohani et al. 2020). It ranks second in agricultural production after cereals (USDA 2018). The production of high-value vegetable oil and protein-rich animal feed, as well as its role as a break crop in cereal-dominated cropping systems makes canola an important crop globally (Angus et al. 2015; Emery et al. 2021). Due to the presence of multiple ecotypes, including spring, semi-seasonal, and winter varieties, it is grown all over the world in various climatic conditions (Batoool et al. 2022). However, drought seriously affects canola productivity, as this crop is deemed as highly susceptible to water shortage at every growth stage, from the initial germination to the final ripening stage (Khanzada et al. 2020). In Tunisia, the introduction of this crop began in 2014 (Medimagh et al. 2018), especially in the northern regions of the country with a production that in 2022 has reached more than 15,000 ha (Maghreb Oilseeds 2022). This crop is cultivated as monoculture and it is very demanding in nitrogen inputs (Zheng et al. 2020; Emery et al. 2021; Madsen et al. 2022). To cover *B. napus* nutritional needs, an excessive use of chemical inputs has been adopted, contributing to negatively impact not only human health (Tudi et al. 2021) but also the environment, increasing biodiversity loss and reducing water and soil quality (Yan et al. 2024). Long-term use of synthetic nitrogen fertilizers can lead to an increase in greenhouse gas emissions such as nitrous oxides inhibition of soil enzyme activity of the soil, with negative effects on soil nitrogen and carbon cycles, and to water pollution from nitrogen losses through run-off and leaching (Zeng et al. 2016; Yan et al. 2017). These environmental consequences are further exacerbated by the intensification of traditional agricultural practices (Martin-Guay et al. 2018), which have prompted increasing regulatory attention (Brandes and Heimbach 2018; Arthey 2020; Zheng et al. 2020). Despite the presence of these strict regulations, new ways by using sustainable crop protection methods may further improve canola eco-friendly production.

Intercropping, an agricultural practice that allows the simultaneous growth of multiple crop species in a single field, has been widely practiced due to its economic, ecological, and environmental benefits (Martin-Guay et al.; Dowling et al. 2021). Establishing intercropping systems based on legumes is a useful strategy to combine increased productivity

T. Reitz

Martin Luther University Halle-Wittenberg (MLU),
Institute of Agricultural and Nutritional Sciences - Crop
Research Unit, Julius-Kühn-Straße 23, 06112 Halle,
Germany

R. Balestrini

National Research Council of Italy, Institute of Biosciences and
Bioresources (IBBR), Bari, Italy

and reduced use of synthetic N fertilizers, mainly due to the ability of legume-associated rhizobia in fixing N₂ (Chai et al. 2021). Furthermore, this practice has significant benefits, such as cultivating more crops simultaneously and allowing to reach the same yield levels as in monoculture, improving soil N and P content, enhancing C sequestration and storage, or promoting beneficial microorganisms growth (Hassen et al. 2017; Hei et al. 2021; Cuartero et al. 2022). Specifically, it has been reported that intercropping may promote functional and architectural alterations to the soil microbiota (Duchene et al. 2017; Yu et al. 2018). In the intercropping system, the soil, the plants and the associated microorganisms, interact with each other in various ways in the so-called plant–microorganism–environment (Zhou et al. 2023). The benefits of intercropping have been attributed to both above-ground interactions among plants, such as improved light absorption and temperature regulation, and below-ground interactions, including root communication and interactions between roots and beneficial soil microorganisms (Brooker et al. 2015; Xue et al. 2016). Intercropping is known to impact on soil health, increasing plant residues and root exudates production and, consequently, improving soil microbial biodiversity (Zhang et al. 2019). Microorganisms have an important role in maintaining ecosystem functionality, influencing soil nutrient turnover by decomposing soil organic matter (Zhou et al. 2023).

Legumes are of special interest in intercropping with *B. napus*, minimizing plant competition for soil, due to the presence of the two different crop species characterized by a different root morphology and nutritional needs in the same space and, therefore, differentially exploiting it (Verret et al. 2017; Dowling et al. 2021). Zhou et al. (2019) reported that the intercropping of *B. napus* with the legume species *Astragalus sinicus* L. promoted the growth of plants and improved crop yield. Moreover, legumes are capable to form symbiotic relationships with N₂-fixing bacteria that are hosted in specialized root structures known as nodules (Xue et al. 2025). Thanks to the presence of these bacteria, legumes are able to improve soil available N in the intercropping system, releasing it in the environment when nodules decompose, allowing the adsorption by other plants (Madsen et al. 2022). In addition, the exudation of soluble N compounds by legumes, followed by uptake by non-legumes, plays a significant role in legume-based agricultural cropping systems. This

process reduces the use of synthetic inorganic N fertilizers and thus supports sustainable agriculture (Thilakarathna et al. 2016; Ilahi et al. 2025). Considering that canola is not able to form symbiotic relationships with N₂-fixing bacteria, its intercropping with legumes improves canola N-adsorption, reducing fertilizers use (Verret et al. 2017; Dowling et al. 2021). However, recent studies highlight the importance of selecting the appropriate legume species in intercropping systems, as outcomes largely depend on the specific crop combination (Raza et al. 2023, 2024). Many studies have emphasized the sustainable re-introduction of legumes into crop systems, given their positive effects on yield, quality, and subsequent crops (Preissel et al. 2015; St. Luce et al. 2015; Stagnari et al. 2017). In particular, intercropping *B. napus* with legumes is considered a sustainable practice that improves yield stability and reduces reliance on chemical inputs, mainly due to biological nitrogen fixation that enhances N use efficiency and mitigates competition for soil and fertilizer-derived N (Verret et al. 2017; Dowling et al. 2021). Moreover, while intercropping modifies microbial taxonomic interactions, its overall impact on soil microbial composition remains limited (Li and Wu 2018).

Accordingly, we hypothesized that intercropping *B. napus* with different legume species (*Medicago sativa* L. vs. *Hedysarum coronarium*) would differentially influence crop performance and the composition of root and rhizosphere associated bacterial communities. To test this, a two-year field experiment was conducted in northwestern Tunisia, comparing canola monoculture with intercropping using two forage legumes: *M. sativa*., widely adopted in intercropping and rotations, and *H. coronarium*, less commonly utilized. This design allowed us to assess whether legume-based intercropping improves canola growth, promotes beneficial microbial communities, and enhances both productivity and soil health relative to monoculture.

Materials and methods

Experimental design

The two years field trial was carried out at the Higher School of Agriculture Education of Mateur in

Bizerte, northwest of Tunisia (37°03'N, 9°36'E.) during canola growing season, from 20 December 2021 to 30 May 2022 and from 29 November 2022 to 25 May 2023, for the first and second year of experiment, respectively. The region is characterized by a sub humid climate, where the conditions are favorable for this crop.

For the experiment, three cropping systems were set up on December 20, 2021 and November 29, 2022, including *Brassica napus* L. variety Y84 grown as sole crop (BN, used as control), *B. napus*

L. intercropped in strips with a Tunisian ecotype of *Hedysarum coronarium* L. (IH) and *B. napus* L. intercropped with *Medicago sativa* L. variety SIRIVER (IM).

We used a randomized complete block design with three treatments (Fig. SW A-B-C) and three replicates, for nine parcels (Figure SZ). Each intercropping system was arranged using three strips of 1.2 m width, and the total area of each parcel was 3.6 m × 5 m. In the intercropping with legumes, *M. sativa* and *H. coronarium* were alternated in each plot using *B. napus*. The distance between plots was 1 m, while, inside each plot, the distance between each line was 20 cm. *B. napus* L. (5 kg/ha), *H. coronarium* L. (60 kg/ha) and *M. sativa* L. (40 kg/ha) were sown with a broadcast seeder due to the small seeds and high target plant density to achieve satisfactory ground cover. During plants growth, no pesticides, fertilizer or irrigation was applied. Weeding was done manually and kept uniform in all treatments. Climatic data, including monthly temperature (minimum, maximum and average, °C), cumulative monthly precipitations (mm), and average monthly relative humidity (%), were obtained from the meteorological station located in the Higher School of Agriculture Education of Mateur from December 2021 to June 2023.

Soil sampling for chemical-physical analysis

Before the beginning of the experiment, soil samples were randomly collected by using a soil sampler through (from 0 to 15 cm depth). After removing large debris, samples were air-dried, and used for standard soil physical–chemical analysis. The measure of soil pH, electrical conductivity (EC, mS/cm), soil organic matter (SOM, %), total nitrogen (TN, %), exchangeable potassium (K, mg/L), exchangeable calcium (Ca, mg/g) exchangeable sodium (Na, mg/g), exchangeable magnesium (Mg, mg/L) and available

phosphorous (P, µg/g) was performed at the Research and Technology Transfer Support Unit in the Centre of Biotechnology of Borj Cedria as described by Gritli et al. 2023.

Measurement of agronomic parameters in *B. napus*

Different agronomic parameters were measured during the two growing phases of *B. napus*, including plant density (plant/m², measured in a square of 0.25 m²), plant height (cm), ramification number (n), number of siliques for plant (n), average number of seeds for silique (n) and thousand kernel weights (TKW). Yield (kg/ha), was estimated as (plants/m²) × (siliques per plant) × (seeds per silique) × (TKW/1000).

Specifically, plant density was recorded after two months from the sowing. At flowering stage, plant height and ramifications number were recorded, as well as the sampling of *B. napus* roots and rhizosphere for bacterial communities' assessment was performed. The number of siliques for plant and the average number of seeds for silique were calculated before harvesting.

Five plants for each parcel, for a total of 15 replicates for each Main plot, were sampled for roots and rhizosphere metabarcoding analysis at the flowering stage in March 2022 and March 2023 for each year, respectively. Plants were randomly selected avoiding borders in each parcel and roots were separated from the aerial part of the plant. Rhizospheric soil was collected as the fraction tightly adhering to the roots after gentle shaking; it was carefully brushed off and stored. The samples were then lyophilized and sieved (2 mm) to remove rocks, root fragments, and large debris, yielding approximately 15 g per sample. Collected roots were cleaned with distilled water and dried with sterile paper and then pulverized with liquid nitrogen for a total amount of 4 g for each sample and stored at −20 °C along with the rhizospheric soil samples.

DNA extraction, sequencing and metabarcoding analysis on roots and rhizospheric soil samples

The bacterial DNA was extracted from about 500 mg of each rhizosphere soil sample by using the Fast DNA SPIN KIT FOR SOIL (OBEXO SCIENTIFIC). The DNA from root samples (100 mg) was extracted

using the GRS Genomic DNA Kit PLANT (Boca Scientific Inc). The protocol was modified by increasing the incubation time from 10 min to 2 h. The DNA was then stored at -20°C . Prior performing PCR, the concentrations of DNA extracts were adjusted from 10 to 15 ng/ml. The amplification of the bacterial 16S rRNA gene V4 region was performed with the universal primers 515f and 806r, which were equipped with Illumina adapter sequences. To ensure correct amplification of the sequences, all PCRs were performed using proofreading KAPA HiFi polymerase (KAPA Biosystems, Boston, MA, USA). The conditions of the PCR are summarized in Table S1. Success of PCR was checked with agarose gel electrophoresis. Amplicons from the PCR-triplicates were pooled and purified with AmpPure XP Beads, indexed in an additional PCR (Illumina Nextera XT v2 index primers) and purified again with AmpPure XP Beads. Concentration of indexed and purified PCR-products was determined with NanoDrop ND-8000 spectrophotometer. DNA of fungal and prokaryotic amplicons were equimolarly pooled. Exact concentrations of the final pools were determined with Qubit dsDNA-HS Assay and fragment length and Quality were additionally checked with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Amplicon pools were combined for Illumina MiSeq paired-end sequencing. Sample libraries and the control library were diluted and denatured following the MiSeq Illumina kit instructions and injected into an Illumina MiSeq flow-cell for paired-end sequencing. Finally, paired-end sequencing of 2 by 300 bp was implemented on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) at the Department of Soil Ecology of the Helmholtz Centre for Environmental Research (UFZ, Halle, Germany). Metabarcoding analysis was performed through QIIME 2 pipeline (Bolyen et al. 2019). First, bacterial sequences were trimmed using cutadapt v3.4, denoised through dada2 v. 2024.10.0 and then assembled into Amplicon Sequence Variants (ASVs). The Silva v. 138.99 (Quast et al. 2013) database was used to train Naive Bayes classifier on ASVs (99% identity) sequences for bacteria taxonomic assignment. Mitochondria and chloroplast were filtered out with the “filter-features” parameter of QIIME 2 from the ASV table. The relative abundance (%) of each ASV in the total amounts of the entire sample was obtained by elaborating the QIIME2 output. Raw reads from metabarcoding of root and rhizosphere samples were submitted to NCBI SRA

under BioProject PRJNA1285884. Taxa visualization and the relative statistical assessment was performed using the ASV table as input for Microbiome Analyst (Chong et al. 2020; Dhariwal et al. 2017). To perform diversity evaluation within samples at feature-level (alpha diversity) the Chao1 and Shannon indexes were used considering the “treatment” factor, that included both the year and the cultivation system conditions, while the Bray–Curtis index was used to evaluate the diversity among samples (beta diversity). To discriminate diversity at order level between rhizo-sphere and roots associated microbial communities the factor “compartment” *i.e.*, rhizosphere and roots, was used, while diversity within each compartment was evaluated at feature-level considering “year” and “cultivation system” factors. Beta diversity was then reported in a two-dimensional principal coordinates analysis (PcoA). Significant differences in alpha and beta diversity was assessed through 2-way ANOVA and PERMANOVA, respectively. Taxa distribution among the different conditions was visualized through stacked barplots at phylum level. To identify specific differences in taxa abundance in the different cultivation systems, differential abundance analysis of bacterial taxa at order level was conducted using the Analysis of Composition of Microbiomes with Bias Correction (ANCOM-BC; Lin and Peddada 2020) embedded in QIIME2 to assess taxa showing changes between the two intercropping systems (IH and IM) versus monoculture (BN). The command “qiime composition ancombc” was used by setting [–p-for-mula “intercropping system”], [–p-reference-levels “intercropping system:BN”] and [–p-prv-cut 20].

Statistical analysis

The different agronomic parameters analyzed included plant density (plants/m²), plant height (cm), ramifications number (n), average number of siliques for each plant (n), and average number of seeds for each silique. Moreover, thousand kernel weight and the plant production parameters were used to assess the estimated yield for each condition. To evaluate the effect of two main factors “year”, “intercropping system” and their interaction on agronomic parameters, a two-way ANOVA was performed with R v.4.3.2. When a significant difference was detected between years, a further analysis was conducted within data

from each year with one-way ANOVA to investigate the differences among each intercropping system in single growing season. Post-hoc analysis was conducted through Tukey Honestly Significant Difference (HSD) test, with a significance level of $p \leq 0.05$.

Results

Soil physical–chemical analyses and climatic data

The soil chemical-physical analysis revealed that the soil was classified as loamy soil (36.3% loam) with a slightly alkaline pH of 7.43. The electrical conductivity (EC) of 3.23 mS/cm reflected moderate salinity. Exchangeable sodium content was relatively high (22.06 mg/g), as well as the phosphorus content (89.56 μ g/g) and the organic matter content (8.369%). On the other hand, the total nitrogen was relatively low (0.22%). Detailed information is reported in Table S2.

Agronomic parameters

Statistical analysis of the agronomic parameters among the different cultivation systems (BN, IH, IM) and between the years (2022 and 2023) highlighted significant variations between the treatments influenced both by climatic conditions and intercropping practices (Table S3). In the year 2023 *B. napus* plants were characterized by a higher density and Height, compared to 2022 (Fig. 1, Table S3). Moreover, in the year 2023 the estimated yield was significantly higher than in 2022 considering all the intercropping systems (340 ± 254 kg/ha and 1350 ± 1159 kg/ha in 2022 and 2023, respectively, Fig. 2e–f, Table S3). In the intercropping with *H. coronarium*, a significant difference was found between 2022 and 2023, with an increase in estimated yield from 632 ± 106 to 2880 ± 386 kg/ha (Fig. 2). Moreover, the intercropping with *H. coronarium* significantly improved *B. napus* estimated yield and showed higher estimated yield values compared to BN and IM (584 ± 271 , 1756 ± 1214 , 195 ± 157 , for BN, IH and IM, respectively, Fig. 2 e–f, Table S3). Monoculture practice was the one that showed the highest difference in plants Height between the two years, with an average plant Height of $78 \text{ cm} \pm 1.00$ and of $132 \text{ cm} \pm 2$ in 2022 and 2023, respectively (Fig. 1c–d, Table S3).

The intercropping with *M. sativa* showed the lowest average seeds per silique number, compared to the other cultivation system (23 ± 2 , 23 ± 3 , 18 ± 4 , for BN, IH and IM, respectively, Fig. 2c–d, Table S3).

A PCA analysis was performed to highlight the difference among treatments in 2022 and 2023 considering the investigated agronomic parameters and the cultivation system (Fig. 3). A clear separation of all the cultivation systems was observed in 2022, while in 2023 only the intercropping with *H. coronarium* (IH) was clearly separated from the other two systems. In 2022 (Fig. 3a), the strongest variables that allowed data separation were the number of seeds, plant Height, number of siliques and ramifications number, and all these variables similarly contributed to data separation. On the other hand, in 2023 the strongest variables were represented by plant density, number of siliques, TKW and plant height (Fig. 3b).

Analysis of bacterial communities

The total number of obtained bacterial reads in rhizosphere samples was 595,939, of which 305,542 were identified in 2022 and 290,397 in 2023. On the other hand, total number of reads in root samples was 254,968 (173,631 for 2022, 81,337 for 2023). Rhizosphere and root samples showed differences on associated taxa (Fig. 4–5). Specifically, beta diversity considering the “compartment” factor was significant, showing that rhizosphere and root samples were characterized by a different taxa composition ($p = 0.001$, $R^2 = 0.23$, F-value = 16.83, Fig. SV).

Within each compartment, the comparison between the years and the cultivation systems highlighted that most of the differences in terms of relative abundance were related to the year rather than the cultivation system (Fig. 4a–b). Alpha diversity of rhizosphere samples was not significantly different between cultivation systems considering both Chao1 and Shannon indexes (Fig. 4c–d), and the same results were obtained for the root samples (Fig. 5c–d). Rhizosphere was characterized by some variations in taxa composition depending on year. In particular, beta diversity highlighted a significant difference in taxa composition between the two year (p -value = 0.001, $R^2 = 0.17$, F-value = 5.58, Fig. SY.b). Considering the cultivation systems (Fig. SY.a), no significant differences were detected. Beta diversity of root samples highlighted no differences considering both the

Fig. 1 Biometric parameters of *B. napus* in 2022 and 2023 under different cultivation systems. **a–b)** Plant density (plants/m²), **c–d)** height (cm), **e–f)** number of ramifications (n). Different letters indicate significant differences among cultivation systems within each year (Tukey HSD, $p \leq 0.05$). BN = monoculture; IH = intercropping with *H. coronarium*; IM = intercropping with *M. sativa*

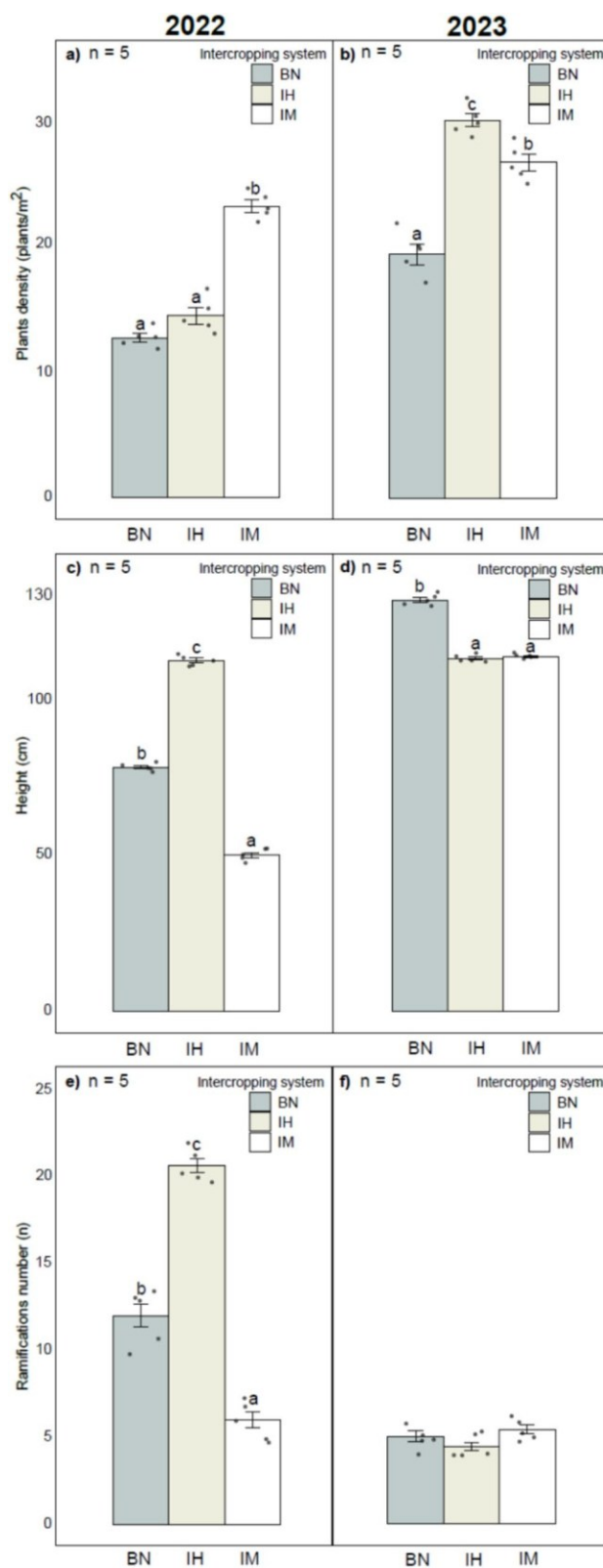
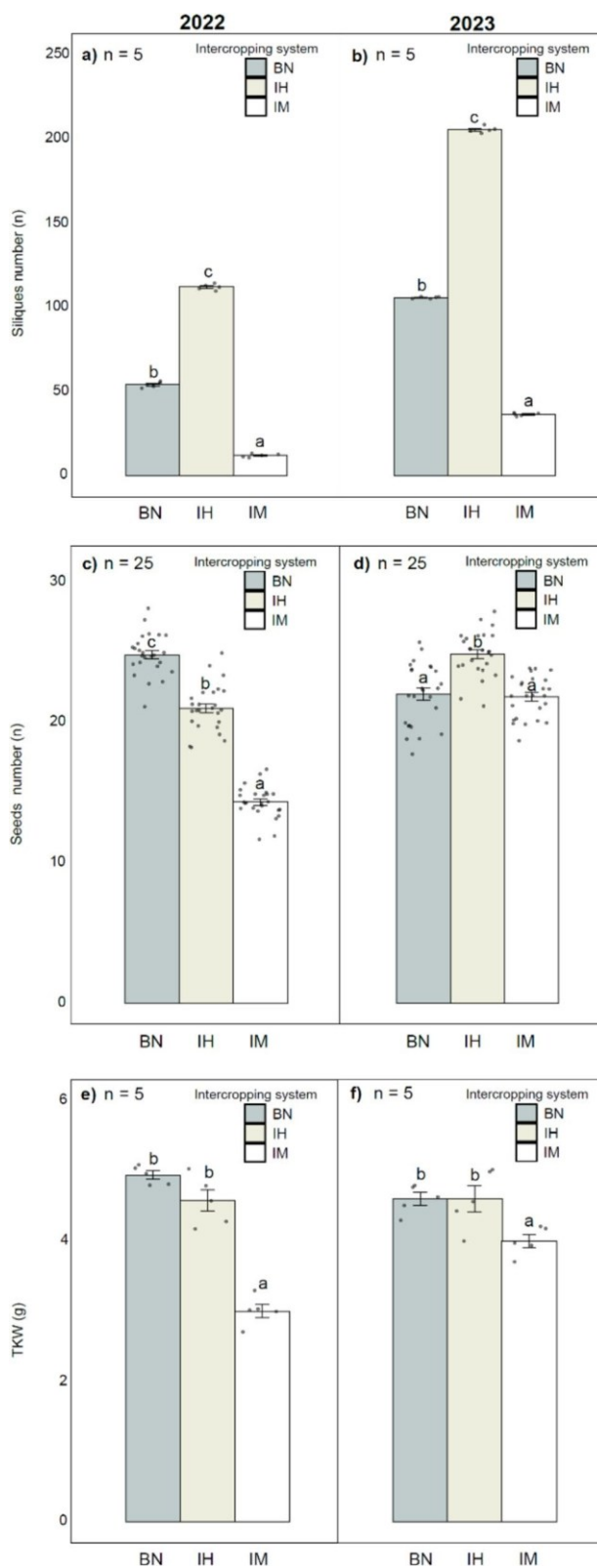


Fig. 2 Production parameters of *B. napus* in 2022 and 2023 under different cultivation systems. **a–b)** Number of siliques, **c–d)** number of seeds per silique, **e–f)** thousand kernel weights (TKW, (g). Different letters indicate significant differences among cultivation systems within each year (Tukey HSD, $p \leq 0.05$). BN = monoculture; IH = intercropping with *H. coronarium*; IM = intercropping with *M. sativa*



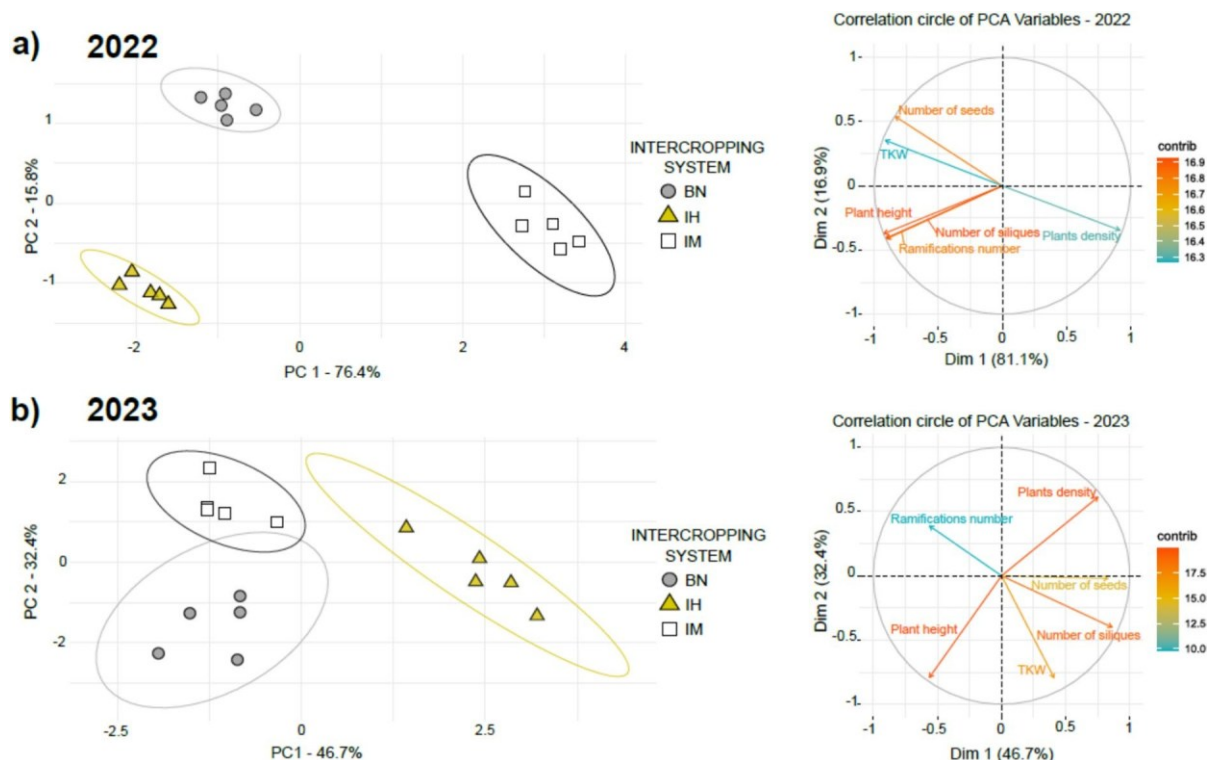


Fig. 3 Principal component analysis of biometric parameters of *B. napus* in 2022 (a) and 2023 (b). Angles $< 90^\circ$ indicate correlated variables, whereas angles $> 90^\circ$ indicate uncorrelated variables. BN = monoculture; IH = intercropping with *H. coronarium*; IM = intercropping with *M. sativa*

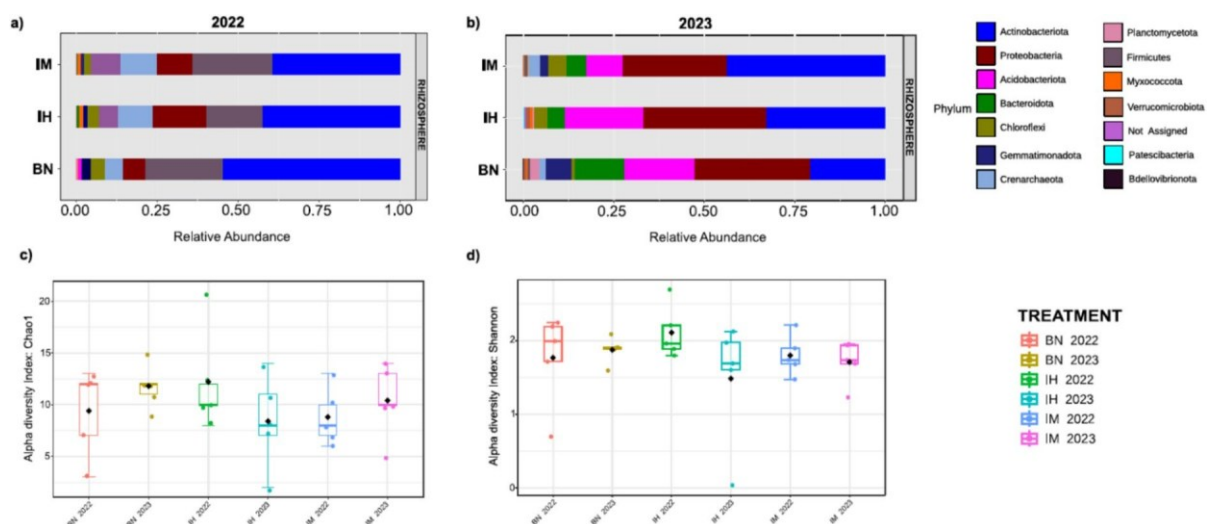


Fig. 4 Bacterial taxa diversity in the rhizosphere in 2022 and 2023. a–b) Taxa bar plots at phylum level. c–d) Alpha diversity of bacterial communities considering both years and inter-cropping systems using Chao1 and Shannon index respectively. BN = monoculture; IH = intercropping with *H. coronarium*; IM = intercropping with *M. sativa*

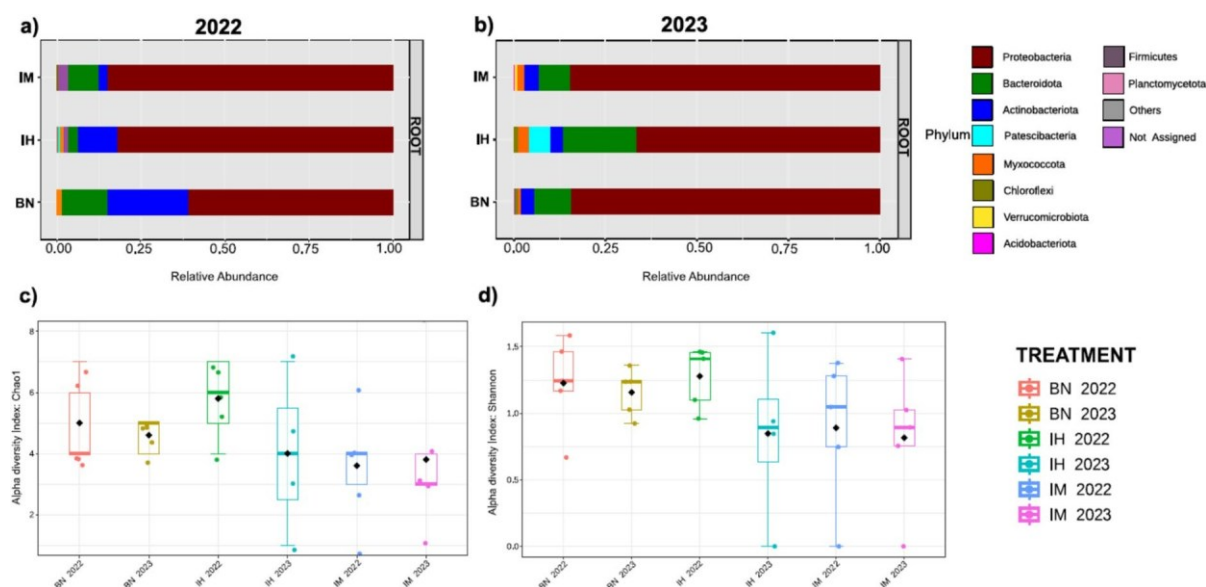


Fig. 5 Bacterial taxa diversity in the roots in 2022 and 2023. **a–b)** Taxa bar plots at phylum level. **c–d)** Alpha diversity of bacterial communities considering both years and intercropping systems using Chao1 and Shannon index respectively. BN = monoculture; IH = intercropping with *H. coronarium*; IM = intercropping with *M. sativa*

“year” and the “cultivation system” factors (Fig. SY c–d).

The rhizosphere of *B. napus* in 2022 showed some variation in taxa relative abundance considering the cultivation system (Fig. 4a). Rhizosphere samples of all cultivation systems (IH, IM and BN) mainly harbored phyla Actinobacteriota, Firmicutes, Proteobacteria (currently assigned as Pseudomonadota by Oren and Garrity 2021), Crenarchaeota, Chloroflexi and Gemmatimonadota (Fig. 4a). Additionally, IH and IM samples were also associated to Bacteroidota and Myxococcota, while BN to Acidobacteriota. In rhizosphere samples in 2023, the core microbiome was similar to that of 2022, including taxa Actinobacteriota, Proteobacteria, Chloroflexi and Gemmatimonadota (Fig. 4b). In 2023, the phylum Acidobacteriota was identified in all samples, as well as the phylum Bacteroidota. As for 2022, in 2023 IH samples were associated with the presence of Myxococcota phylum. BN samples were associated with the presence of Planctomycetota (Fig. 4b). In both years, the monoculture (BN) showed an overall higher number of taxa compared to the other cultivation systems. ANCOM-BC analysis revealed that in the rhizosphere samples, orders Pyrinomonadales, Microtrichales and Chloroflexales significantly increased in IH compared to BN, while orders Bacteriovorales, Xanthomo-

nadales, Micromonosporales, Steroidobacterales, Gemmatimonadales and Rubrobacterales showed the opposite trend (Fig. 6a). Considering IM, the order Pyrinimonadales showed a significant increase compared to BN. As for IH, also in IM orders Bacteriovorales, Gemmatimonadales and Rubrobacterales decreased. The two additional orders showing a decrease in this condition were Longimicrobiales and Tepidisphaerales (Fig. 6b).

Concerning root samples, in 2022, main associated phyla were Proteobacteria, Actinobacteriota and Bacteroidota. Additionally, IM and IH were both characterized by the presence of Myxococcota. On the other hand, Patescibacteria and Planctomycetota were associated exclusively to IH samples (Fig. 5a). In 2023, main associated taxa in roots were Proteobacteria, Bacteroidota, Actinobacteriota and Myxococcota (Fig. 5b). Additionally, IM samples were associated also with Verrucomicrobiota and Acidobacteriota, while IH samples with Patescibacteria and Chloroflexi. The BN samples showed exclusive presence of Chloroflexi phylum.

ANCOM-BC analysis performed on root samples revealed a limited number of differentially abundant taxa among cultivation systems, if compared

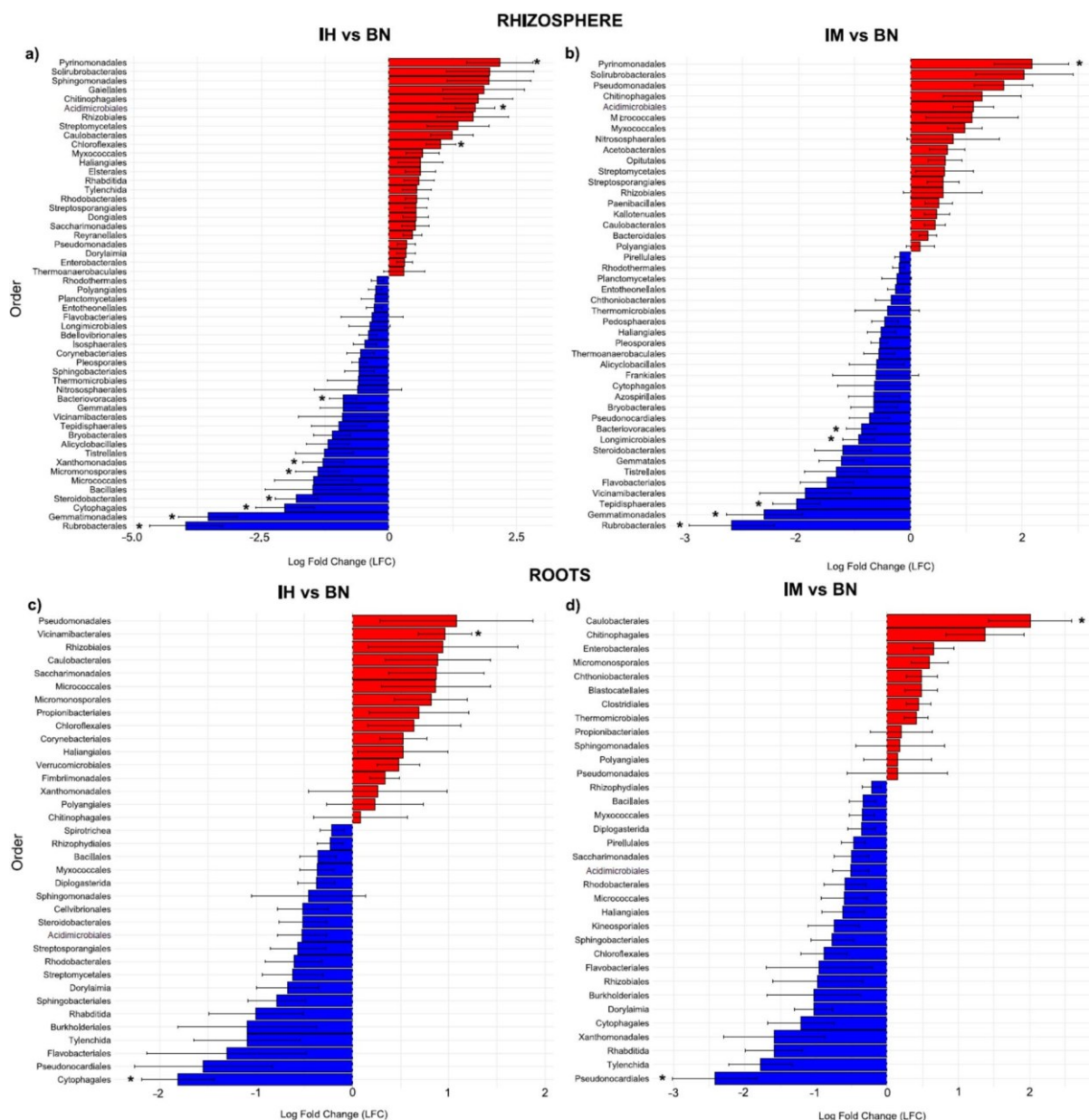


Fig. 6 Differential abundance analysis of bacterial taxa with ANCOM-BC. **a–c)** Orders significantly different in *H. coronarium* intercropping vs. monoculture (rhizosphere and roots). **b–d)** Orders significantly different in *M. sativa* intercropping vs. monoculture (rhizosphere and roots). Bars show log fold change (LFC $\pm$ SE); significant differences marked with “*” ($p \leq 0.05$). BN = monoculture; IH = intercropping with *H. coronarium*; IM = intercropping with *M. sativa*

to outcomes from analysis on the rhizosphere ones (Fig. 6c–d). In particular, in IH, order Vicinamibacteriales increased, while order Cytophagales

decreased compared to BN (Fig. 6c). On the other hand, in IM samples an increase in Caulobacteriales

and a decrease in Pseudonocardiales was observed compared to BN (Fig. 6d).

Discussion

The two intercropped legume species differentially affect *B. napus* agronomic parameters especially in the dry growing season

The scarcity of water adversely affects germination, plant density, mineral uptake, shoot elongation, seed development, yield and quality in *B. napus* (Batool et al. 2022). In our case, the reduced amount of water from rainfall, coincidental with the flowering of *B. napus* during March–April 2022, can likely be the cause of the significant overall reductions in all agronomic parameters in that year compared to 2023. However, particularly in the drought-affected year (2022), the PCA on *B. napus* agronomic parameters clearly shows that intercropping with either *H. coronarium* or *M. sativa* produced a contrasting effect on all major yield components of canola. In both years, canola intercropped with *H. coronarium* showed improved agronomic parameters, compared to the monoculture, while in 2023 the positive effect of intercropping was less pronounced. A study of Leoni et al. (2024) showed that intercropping sorghum with

H. coronarium improved agroeconomic performance of this crop species. In addition, the drought-mitigation effect we observed in 2022 mediated by the intercropping with *H. coronarium* is consistent with the literature. Intercropping may favor water acquisition in drought conditions due to the presence of different crop species that can be characterized by complementary root architectures and, therefore, improving soil root colonization (Brooker et al. 2015). For example, Renwick et al. (2020) showed that maize/pigeon pea intercropping outperformed monocultures during a drought period. The authors attributed the advantage to complementary rooting patterns and legume-mediated facilitation of the cereal under water deficit (Renwick et al. 2020), mechanisms that are plausible for our intercropping system as well.

On the other hand, canola intercropped with *M. sativa* resulted in reduced plant productivity, as TKW and silique number were lower compared to monoculture, especially in 2022. This particular scenario highlights the importance of choosing the correct legume species

to be intercropped with *B. napus*. The worsening of some canola agronomic parameters in plants intercropped with *M. sativa* may be explained by its allelopathic effects on *B. napus* growth. *M. sativa* is known to secrete several allelopathic compounds, including phenolic acids, saponins, flavonoids, alkaloids, and coumarins (Ghimire et al. 2019; Abouzeid et al. 2023). These compounds can be released into the environment through root exudation, volatilization from aboveground tissues, leaching from leaves during rainfall, or decomposition of plant residues (Scavo et al. 2018). Several studies have reported inhibitory effects of *M. sativa* residues on crops: for example, Xuan and Tsuzuki (2002) and Dang Xuan et al. (2005) observed reduced tiller number and yield in barley and decreased plant height in wheat. Similarly, intercropping *Zea mays* with *M. sativa* led to decreased corn grain and total yield (Berti et al. 2021). The adopted strip intercropping system in our experiments may have further enhanced allelopathic interactions, as previously documented (Scavo and Mauromicale 2021). Moreover, abiotic stresses such as drought and high temperatures are known to increase allelochemical production (Scavo and Mauromicale 2021). In our study, the rainfall deficit in 2022 and elevated temperatures, combined with strip intercropping, may have amplified the allelopathic effects of *M. sativa* on *B. napus*, thereby reducing canola productivity. Beyond direct negative effects on plant growth, allelochemicals can also influence soil microbial communities by selectively inhibiting or promoting microbial taxa, thus potentially shaping rhizosphere interactions and nutrient cycling (Zhang et al. 2019; Qu et al. 2021; Revillini et al. 2023).

Rhizosphere and root microbial communities were influenced by the adopted legume species in the intercropping systems

It has been reported that the agricultural practices, including intercropping, may have an impact on soil microbiota diversity (Duchene et al. 2017; Yu et al. 2018). However, considering that no significant differences in alpha diversity of rhizospheric and roots samples were observed in our field trial, it can be argued that, in our experiment, diversity of the soil bacterial community was not affected by the intercropping system. In previous studies carried out in other intercropping systems, only slightly changes

were observed in the soil bacterial community (Bargaz et al. 2017; Mwakilili et al. 2021; Zampieri et al. 2025). Moreover, Madsen et al. (2022) reported no changes in the soil microbial diversity between samples collected from *B. napus* grown as monoculture and intercropped with *Pisum sativum*. It has been suggested that the shift in bacterial communities in *B. napus* seeds can be associated with the environmental conditions rather than the plant host genotype (Morales Moreira et al. 2021). This may explain why the strongest bacterial variations observed in our study are mainly due to the different climatic conditions observed in the two years and less differences were found to be associated with the adopted legume plant species in intercropping system. For example, Acidobacteriota abundance varied consistently to the year. In fact, an increase in this phylum members were observed in 2023 compared to 2022. This result is in agreement with Huber et al. (2022) study that showed that Acidobacteriota decreased under water scarcity conditions, as observed in our trial for 2022 compared to 2023. Moreover, phylum Bacteroidota increased in 2023 compared to 2022. This data is in accordance with what has been reported by Wolińska et al. (2017), that highlighted how this phylum includes several members able to grow in water-rich environments. In fact, 2023 was the year characterized by the heaviest rainfall amount. Also, the phyla Firmicutes, Myxococcota and Actinobacteriota showed variations considering the climatic conditions. In fact, these phyla were more abundant in 2022 under water reduced conditions compared to 2023. The year 2022 was characterized by a lower rainfall amount and several studies reported that members of these phyla are drought-tolerant (Calvo et al. 2025; Dai et al. 2021; Ebrahimi-Zarandi et al. 2023).

Despite the lack of significant differences in the alpha diversity of the rhizosphere and root bacterial communities, we did see differences in the composition of some specific bacterial taxa among the different cultivation systems. This has been previously observed in other legume-intercropped plants, such as melon (Cuartero et al. 2022). Looking in details, Crenarchaeota relative abundance was higher in the two used intercropping systems compared to monoculture. Legumes are known to harbor rhizobia that fix atmospheric N and subsequently produce ammonium (Lai et al. 2022), and Crenarchaeota are able to oxidize ammonia (Schrader et al. 2022). This can explain

their increase under legume-based intercropping. The same scenario has been observed for Patescibacteria in roots of *B. napus* intercropped with *H. coronarium*. In fact, it has been reported that Patescibacteria increased in legume-based intercropping (Liu et al. 2024), specifically in the rhizosphere of the non-legume crop used in the system (Ma et al. 2024).

Interestingly, in our study, the orders Xanthomonadales and Steroidobacterales, members of Gammaproteobacteria, were more abundant in the monoculture rhizosphere than in the legume-intercropped conditions, suggesting these two taxa may be part of the specific microbial core of the species *B. napus*. Particularly, monoculture canola core microbiome harbored *Steroidobacter* genus (Madsen et al. 2022), and members of Gammaproteobacteria were indeed identified in *B. napus* in all its development stages (Taye et al. 2022). In addition, the monoculture showed an overall higher abundance of Gemmatimonadota members compared to the two other intercropping systems. Yang et al. (2025) highlighted that this phylum increased in *B. napus* cultivation under N deficit condition. In our study, it could be argued that the soil surrounding *B. napus* plants grown alone had probably a lower N content compared to the soil of the legumes-based intercropping systems, due to the legumes ability in fixing atmospheric N and to the absence of fertilizers applied in the monoculture condition. However, further chemical analysis on specific soil samples will be needed to clarify this hypothesis.

The rhizosphere of *B. napus* showed that the orders Pyrinomonadales and Acidimicrobiales increased in IH samples, compared to monoculture. Pyrinomonadales order has been associated with P availability in soils, as members of this order possess *pit* genes responsible for P assimilation (Xia et al. 2023). Pyrinomonadales abundance has been reported to increase under legume crop rotation (Xia et al. 2023). The order Acidimicrobiales include bacteria able to fix N (Lopes et al. 2015), therefore their increase in IH system may be related to the presence of this legume, that may have recruited these bacteria in nodules (Ilyas et al. 2022).

In the root compartment, *B. napus* intercropped with IH and IM samples showed that Vicinamibacterales and Caulobacterales orders, respectively, increased with respect to monoculture. Since Vicinamibacterales and Caulobacterales seem to increase under similar condition but under two different

leguminous species, a probable role of legumes in recruiting these specific taxa may be hypothesized. Vicinamibacterales order has been found to be related to nutrient cycle, including P cycle in soils (Wang et al. 2024). Vicinamibacteraceae family has been reported to increase in nutrient rich soils, with positive correlations to N and organic matter (Niu et al. 2024). These results may explain why we observed an increase of these order in IH samples. On the other hand, in IM an increase in Caulobacterales was observed. This order has been detected as one of the main orders in roots of soybean (Yazaki et al. 2021). It has been reported that this order is related to the presence of organic acids in soils (Shi et al. 2011) and to positively correlates with nitrogen and organic matter (Nwachukwu et al. 2022). Interestingly, a link between *Sinorhizobium meliloti*, a nodule producing bacteria associated to *M. sativa*, and an increase in abundance of Caulobacterales has been suggested in *Vicia faba* roots (Benidire et al. 2020). Additionally, Edulamudi et al. (2011) discovered a *Caulobacter* species able to nodulate in presence of a leguminous crop i.e., *Macrotyloma uniflorum*. Based on this information, we may hypothesize that the increase of Caulobacterales member in IM root samples may depend on the possible role of this taxa as nodulating bacteria in *M. sativa* roots. It is also worth considering that while *B. napus* is a non-mycorrhizal species, both *M. sativa* and *H. coronarium* are mycorrhizal hosts. The extended hyphal networks of arbuscular mycorrhizal fungi associated with legumes can enhance nutrient acquisition, alter soil microbial interactions, and indirectly influence the bacterial communities recruited by *B. napus* (Floc'h et al. 2022). Moreover, because the block design was maintained across years, a potential legacy effect of previous plant cultivation on soil microbial communities cannot be excluded (Nannipieri et al. 2023). Such legacy effects may have contributed to some of the inter-annual microbial differences observed in our experiment and should be taken into account when interpreting intercropping effects.

Conclusions

Our study highlights that the choice of legume species in intercropping systems strongly influences both the productivity of *B. napus* and the composition of

its associated microbial communities. Intercropping with *H. coronarium* improved agronomic performance, especially under drought conditions, compared to both monoculture and intercropping with *M. sativa*, which negatively affected productivity. Climatic conditions also shaped plant performance and microbial community structure, but cultivation system played a key role. Specific bacterial taxa in the rhizosphere and root compartments, such as Crenarchaeota, Patescibacteria, Vicinamibacterales, and Caulobacterales, were enriched depending on the legume used. These findings highlight the importance of selecting suitable legume species to enhance crop yield and support beneficial microbial communities.

Acknowledgements The authors thank Beatrix Schnabel from the Department of Soil Ecology (UFZ) for the great support in the sequencing analysis. All contributions made by members of the Centre of biotechnology of Borj Cedria were sincerely appreciated. We gratefully acknowledge the late Dr. Slim SLIM, former Associate Professor at the Higher School of Agriculture of Mateur, whose valuable guidance during the early stages of this research contributed significantly to its development.

Funding The work was partially funded by the PRIMA RESCHEDULE project (Italian MUR DD 1293/2021, German BMBF 01DH21019 and Tunisian PRIMA/TN/20/06).

Data Availability The raw sequencing reads from metabarcoding of root and rhizosphere samples generated during this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1285884.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

- Abouzeid S, Beutling U, Elekhaw E, Selmar D (2023) Antibacterial and antibiofilm effects of allelopathic compounds identified in *Medicago sativa* L. seedling exudate against *Escherichia coli*. *Molecules* 28:2645. <https://doi.org/10.3390/molecules28062645>
- Angus JF, Kirkegaard JA, Hunt JR, Ryan MH, Ohlander L, Peoples MB (2015) Break crops and rotations for wheat. *Crop Pasture Sci* 66:523–552. <https://doi.org/10.1071/CP14252>

- Arthey T (2020) Challenges and perspectives in global rape-seed production www.agribenchmark.org/cash-crop challenges and perspectives in global rapeseed production
- Bargaz A, Noyce GL, Fulthorpe R, Carlsson G, Furze JR, Jensen ES, Dhiba D, Issac ME (2017) Species interactions enhance root allocation, microbial diversity and P acquisition in intercropped wheat and soybean under P deficiency. *Appl Soil Ecol* 120:179–188. <https://doi.org/10.1016/j.apsoil.2017.08.011>
- Batool M, Mahmoud Ali El-Badri A, Hassan M, Haiyun Y, Wang C, Zhenkun Y, Jie K, Wang B, Zhou G (2022) Drought stress in *Brassica napus*: effects, tolerance mechanisms, and management strategies. *J Plant Growth Regul* 42:1–25. <https://doi.org/10.1007/s00344-021-10542-9>
- Benidire L, El Khalloufi F, Oufdou K, Barakat M, Tulumello J, Ortet P, Heulin T, Achouak W (2020) Phytobeneficial bacteria improve saline stress tolerance in *Vicia faba* and modulate microbial interaction network. *Sci Total Environ* 729:139020. <https://doi.org/10.1016/j.scitotenv.2020.139020>
- Berti MT, Cecchin A, Samarappuli DP, Patel S, Lenssen AW, Moore KJ, Wells SS, Kazula MJ (2021) Alfalfa established successfully in intercropping with corn in the Mid-west US. *Agronomy* 11:1676. <https://doi.org/10.3390/agronomy11081676>
- Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwards CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolk T, Kreps J, Langille MGI, Lee J, Ley R, Liu YX, Lofffield E, Lozupone C, Maher M, Marotz C, Martin BD, McDonald D, McIver LJ, Melnik AV, Metcalf JL, Morgan SC, Morton JT, Naimey AT, Navas-Molina JA, Noltias LF, Orchanian SB, Pearson T, Peoples SL, Petras D, Preuss ML, Priesse E, Rasmusen LB, Rivers A, Robeson MS, Rosenthal P, Segata N, Shaffer M, Shiffer A, Sinha R, Song SJ, Spear JR, Swafford AD, Thompson LR, Torres PJ, Trinh P, Tripathi A, Turnbaugh PJ, Ul-Hasan S, van der Hooft JJJ, Vargas F, Vázquez-Baeza Y, Vogtmann E, von Hippel M, Walters W, Wan Y, Wang M, Warren J, Weber KC, Williamson CHD, Willis AD, Xu ZZ, Zaneveld JR, Zhang Y, Zhu Q, Knight R, Caporaso JG (2019) Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37:852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Brandes M, Heimbach U (2018) Pyrethroid resistance of insect pests of oilseed rape in Germany. *IOBC WPRS Bull* 136:6972
- Brooker RW, Bennett AE, Cong WF, Daniell TJ, George TS, Hallett PD, Hawes C, Iannetta PPM, Jones HG, Karley AJ, Li L, McKenzie BM, Pakeman RJ, Paterson E, Schöb C, Shen J, Squire G, Watson CA, Zhang C, Zhang F, Zhang J, White PJ (2015) Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytol* 206:107–117. <https://doi.org/10.1111/nph.13132>
- Calvo A, Reitz T, Sillo F, Montesano V, Cañizares E, Zampieri E, Mahmoudi R, Gohari G, Chitarra W, Giovannini L, Conte A, Mennone C, Petruzzelli G, Centritto M, González-Guzmán M, Arbona V, Fotopoulos V, Balestrini R (2025) Interactions between an arbuscular mycorrhizal inoculum and the root-associated microbiome in shaping the response of *Capsicum annuum* “Locale di Senise” to different irrigation levels. *Plant Soil* 508:361–383. <https://doi.org/10.1007/s11104-024-06806-4>
- Chai Q, Nemecek T, Liang C, Zhao C, Yu A, Coulter JA, Wang Y, Hu F, Wang L, Siddique KHM, Gan Y (2021). Integrated farming with intercropping increases food production while reducing environmental footprint. *Proc Natl Acad Sci USA* 118. <https://doi.org/10.1073/PNAS.2106382118/-/DCSUPPLEMENTAL>
- Chong J, Liu P, Zhou G, Xia J (2020) Using microbiome analyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Protoc* 15:799–821. <https://doi.org/10.1038/s41596-019-0264-1>
- Cuartero J, Pascual JA, Vivo JM, Ozbolat O, Sanchez-Navarro V, Egea Cortines M, Zornoza R, Mena M, Garcia E, Ros M (2022). A first-year melon/cowpea intercropping system improves soil nutrients and changes the soil microbial community. *Agric Ecosyst Environ* 328. <https://doi.org/10.1016/J.AGEE.2022.107856>
- Dai Y, Pan Y, Sun Y, Zeng J, Liu G, Zhong W, Li X, Wu Y, Lin X (2021) Moisture effects on the active prokaryotic communities in a saline soil unraveled by 18O-informed metagenomics. *J Soils Sediments* 21:430–440. <https://doi.org/10.1007/s11368-020-02771-4>
- Dang Xuan T, Tawata S, Khanh T, Chung I (2005) Decomposition of allelopathic plants in soil. *J Agron Crop Sci* 191:162–171. <https://doi.org/10.1111/j.1439-037X.2005.171>
- Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J (2017) Microbiome analyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res* 45:W180. <https://doi.org/10.1093/nar/gkx295>
- Dowling A, Sadras O, Roberts P, Doolette A, Zhou Y, Den-ton MD (2021) Legume-oilseed intercropping in mechanised broadacre agriculture – a review. *Field Crop Res* 260:107980. <https://doi.org/10.1016/j.fcr.2020.107980>
- Duchene O, Vian JF, Celette F (2017) Intercropping with legume for agroecological cropping systems: complementarity and facilitation processes and the importance of soil microorganisms. A review. *Agric Ecosyst Environ* 240:148–161. <https://doi.org/10.1016/j.agee.2017.02.019>
- Ebrahimi-Zarandi M, Etesami H, Glick BR (2023) Fostering plant resilience to drought with Actinobacteria: unveiling perennial allies in drought stress tolerance. *Plant Stress* 10:100242. <https://doi.org/10.1016/j.stress.2023.100242>
- Edulamudi P, Antony Masilamani AJ, Divi VRSG, Konada VM (2011) Novel root nodule bacteria belonging to the genus *Caulobacter*. *Lett Appl Microbiol* 53:587–591. <https://doi.org/10.1111/j.1472-765X.2011.03151.x>
- El-Badri AM, Batool M, Mohamed I, Wang Z, Khatab A, Sherif A, Ahmad H, Khan MN, Hassan HM, Elrewainy IM, Kuai J, Zhou G, Wang B (2021) Antioxidative and

- metabolic contribution to salinity stress responses in two rapeseed cultivars during the early seedling stage. *Anti-oxidants* 10:1227–1248
- Emery SE, Anderson P, Carlsson G, Friberg H, Larsson MC, Wallenhammar AC, Lundin O (2021) The potential of intercropping for multifunctional crop protection in oil-seed rape (*Brassica napus* L.). *Front Agron*. <https://doi.org/10.3389/fagro.2021.782686>
- Floc'h JB, Hamel C, Laterrière M, Tidemann B, St-Arnaud M, Hijri M (2022) Long-term persistence of arbuscular mycorrhizal fungi in the rhizosphere and bulk soils of non-host *Brassica napus* and their networks of co-occurring microbes. *Front Plant Sci* 13:828145. <https://doi.org/10.3389/fpls.2022.828145>
- Ghimire BK, Ghimire B, Yu CY, Chung IM (2019) Allelopathic and autotoxic effects of *Medicago sativa*-derived allelochemicals. *Plants* 8(7):233. <https://doi.org/10.3390/plants8070233>
- Gritli T, Ellouze W, Wu H, Taamali W, Fu W, Alfeddy MN, Ouahmane L, Courty PE, Wipf D, El Idrissi MM, Chen B, Mnasri B (2023) Molecular characterization of arbuscular mycorrhizal communities associated with *Lathyrus cicera* L. grown in northern Tunisia soils. *Symbiosis* 90:81–90. <https://doi.org/10.1007/s13199-023-00922-y>
- Hassan MU, Chattha MU, Khan I, Chattha MB, Barbanti L, Aamer M, Iqbal MM, Nawaz M, Mahmood A, Ali A, Aslam MT (2021) Heat stress in cultivated plants: nature, impact, mechanisms, and mitigation strategies—a review. *Plant Biosyst Int J Dealing All Aspects Plant Biol* 155:211–234. <https://doi.org/10.1080/11263504.2020.1727987>
- Hassen A Talore D, Tesfamariam E, Friend M, Mpanza T (2017) Potential use of forage-legume intercropping technologies to adapt to climate change impacts on mixed crop-livestock systems in Africa: a review. *Reg Environ Change* 73. <https://doi.org/10.1007/s10113-017-1131-7>
- Hei Z, Xiang H, Zhang J, Liang K, Zhong J, Li M, Lu Y (2021) Rice intercropping with water mimosa (*Neptunia oleracea* Lour.) can facilitate soil N utilization and alleviate apparent N loss. *Agric Ecosyst Environ* 313:107378. <https://doi.org/10.1016/J.AGEE.2021.107378>
- Huber KJ, Vieira S, Sikorski J, Wüst PK, Fösel BU, Gröngroft A, Overmann J (2022) Differential response of Acidobacteria to water content, soil type, and land use during an extended drought in African savannah soils. *Front Microbiol*. <https://doi.org/10.3389/fmicb.2022.750456>
- Ilahi H, Calvo A, Dhane S, Missbah El Idrissi M, Ouahmane L, Alfeddy MN, Reitz T, Sillo F, Balestrini R, Mnasri B (2025) Impact of bacterial inoculations on *Pisum sativum* L. biometric parameters and associated bacterial and AM fungal communities under semi-arid field conditions in Tunisia. *Appl. Soil Ecol*. 205:105757. <https://doi.org/10.1016/j.apsoil.2024.105757>
- Ilyas N, Yang Y, Zhang C, Singh RP, Yu Q, Yuan Y, You X, Li Y (2022) Temporal dynamics and variation in the alfalfa root nodule and rhizosphere microbial communities of coastal sand and lawn soil. *J Plant Interact* 17:173–182. <https://doi.org/10.1080/17429145.2021.2024899>
- Khanzada H, Wassan GM, He H, Mason AS, Keerio AA, Khanzada S, Faheem M, Solangi AM, Zhou Q, Fu D, Huang Y, Rasheed A (2020) Differentially evolved drought stress indices determine the genetic variation of *Brassica napus* at seedling traits by genome-wide association mapping. *J Adv Res* 24:447–461. <https://doi.org/10.1016/j.jare.2020.05.019>
- Kogan F, Guo W, Yang W (2019) Drought and food security prediction from NOAA new generation of operational satellites. *Geomat Nat Hazards Risk* 10:651–666. <https://doi.org/10.1080/19475705.2018.1541257>
- Lai H, Gao F, Su H, Zheng P, Li Y, Yao H (2022) Nitrogen distribution and soil microbial community characteristics in a legume–cereal intercropping system: a review. *Agronomy* 12:1900. <https://doi.org/10.3390/agronomy12081900>
- Leoni F, Lazzaro M, Carlesi S, Moonen AC (2024) Screening suitable legumes for living mulches to support nitrogen dynamics and weed control in a durum wheat–forage sorghum crop sequence. *Field Crops Research* 307, 109246. <https://doi.org/10.1016/j.fcr.2023.109246>
- Li S, Wu F (2018) Diversity and co-occurrence patterns of soil bacterial and fungal communities in seven intercropping systems. *Front Microbiol*. <https://doi.org/10.3389/fmicb.2018.01521>
- Lin H, Peddada SD (2020) Analysis of compositions of microbiomes with bias correction. *Nat Commun* 11:3514. <https://doi.org/10.1038/s41467-020-17041-7>
- Liu J, Zhang W, Teng C, Pang Z, Peng Y, Qiu J, Lei J, Su X, Zhu W, Ding C (2024) Intercropping changed the soil microbial community composition but no significant effect on alpha diversity. *Front Microbiol*. <https://doi.org/10.3389/fmicb.2024.1370996>
- Lohani N, Jain D, Singh M B, Bhalla P L (2020) Engineering multiple abiotic stress tolerance in Canola, *Brassica napus*. *Front Plant Sci*. <https://doi.org/10.3389/fpls.2020.00003>
- Lopes AR, Bello D, Prieto-Fernández Á, Trasar-Cepeda C, Manaia CM, Nunes OC (2015) Relationships among bulk soil physicochemical, biochemical, and microbiological parameters in an organic alfalfa-rice rotation system. *Environ Sci Pollut Res* 22:11690–11699. <https://doi.org/10.1007/s11356-015-4410-1>
- Ma H, Zhou J, Ge J, Zamanian K, Wang X, Yang Y, Zeng Z, Zhao B, Hu Y, Zang H (2024) Oat/soybean intercropping reshape the soil bacterial community for enhanced nutrient cycling. *Land Degrad Dev* 35:5200–5209. <https://doi.org/10.1002/ldr.5290>
- Madsen IJ, Parks JM, Friesen ML, Clark RE (2022) Increasing biodiversity and land-use efficiency through pea (*Pisum sativum*)-canola (*Brassica napus*) intercropping (Peaola). *Front Soil Sci*. <https://doi.org/10.3389/fsoil.2022.818862>
- Maghreb O (2022) Présentation du programme Maghreb Oléagineux Tunisie. Available at: <http://maghreboleagineux.com/le-programme/>. Accessed 26 Apr 2025
- Martin-Guay MO, Paquette A, Dupras J, Rivest D (2018) The new green revolution: sustainable intensification of agriculture by intercropping. *Sci Total Environ* 615:767–772. <https://doi.org/10.1016/j.scitotenv.2017.10.024>
- Medimagh S, Mechri M, Mastouri M, Ben H, Khamassi K (2018) Genetic variability studies for morphological and qualitative attributes among spring rapeseed (*Brassica napus* L.) varieties under rainfed conditions of Tunisia. In: proceedings international congress on oil and protein

- crops. Moldova Academy of Science, Chisinau, Moldova. 20–24 May 2018 41
- Morales Moreira ZP, Helgason BL, Germida JJ (2021) Environment has a stronger effect than host plant genotype in shaping spring *Brassica napus* seed microbiomes. *Phytobiomes J* 5:220–230. <https://doi.org/10.1094/PBIOM-ES-08-20-0059-R>
- Mwakilili AD, Mwaikono KS, Herrera SL, Midega CAO, Mag-ingo F, Alsanius B, Dekker T, Lyantagaye SL (2021) Long-term maize-Desmodium intercropping shifts structure and composition of soil microbiome with stronger impact on fungal communities. *Plant Soil* 467:437–450. <https://doi.org/10.1007/s11104-021-05082-w>
- Nannipieri P, Hannula SE, Pietramellara G, Schloter M, Sizmur T, Pathan SI (2023) Legacy effects of rhizodeposits on soil microbiomes: a perspective. *Soil Biol Bio-chem* 184:109107. <https://doi.org/10.1016/j.soilbio.2023.109107>
- Niu H, Yuan M, Chen X, Zhao J, Cui Y, Song Y, Zhou S, Song A, Huang Y (2024) Deciphering the differences of bacterial communities between high- and low-productive wheat fields using high-throughput sequencing. *Front Microbiol*. <https://doi.org/10.3389/fmicb.2024.1391428>
- Nwachukwu BC, Ayangbenro AS, Babalola OO (2022) Effects of soil properties and carbon substrates on bacterial diversity of two sunflower farms. *AMB Expr* 12:47. <https://doi.org/10.1186/s13568-022-01388-9>
- Oren A, Garrity G M (2021) Valid publication of the names of forty-two phyla of prokaryotes. *Int J Syst Evol Microbiol* 71. <https://doi.org/10.1099/ijsem.0.005056>
- Preissel S, Reckling M, Schläpke N, Zander P (2015) Magnitude and farm-economic value of grain legume precrop benefits in Europe: a review. *Field Crop Res* 175:64–79. <https://doi.org/10.1016/j.fcr.2015.01.012>
- Qu T, Du X, Peng Y, Guo W, Zhao C, Losapio G (2021) Invasive species allelopathy decreases plant growth and soil microbial activity. *PLoS ONE* 16:e0246685. <https://doi.org/10.1371/journal.pone.0246685>
- Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41:D590–D596. <https://doi.org/10.1093/nar/gks1219>
- Rahmani R, Neji M, Belgacem AO, Debouba M (2020) Potential distribution and the habitat suitability of the African mustard (*Brassica tournefortii*) in Tunisia in the context of climate change. *Arab J Geosci* 13:512. <https://doi.org/10.1007/s12517-020-05467-8>
- Raza MA, Zhiqi W, Yasin H, Gul H, Qin R, Rehman S, Mahmood A, Iqbal Z, Ahmed Z, Luo S, Juan C, Liang X, Gitari H, Khalid MH, Feng L, Zhongmin M, Moussa A (2023) Effect of crop combination on yield performance, nutrient uptake, and land use advantage of cereal/legume intercropping systems. *Field Crops Res* 304:109144. <https://doi.org/10.1016/j.fcr.2023.109>
- Raza MA, Mohi, Ud Din A, Shah GA, Zhiqi W, Feng L, Gul H, Yassin HS, Rehman S, Juan C, Liang X, Rehman R, Garawi AMA, Werf W, Qin R, Xin L, Khalid MH, Zhong-min M (2024) Legume choice and planting configuration influence intercrop nutrient and yield gains through complementarity and selection effects in legume-based wheat intercropping systems. *Agric Syst* 220(October 2024):104081. <https://doi.org/10.1016/j.agry.2024.104081>
- Renwick LL, Kimaro AA, Hafner JM, Rosenstock TS, Gaudin AC (2020) Maize-pigeonpea intercropping outperforms monocultures under drought. *Front Sustain Food Syst* 4:562663
- Revillini D, David AS, Reyes AL, Knecht LD, Vigo C, Allen P, Searcy CA, Afkhami ME (2023) Allelopathy-selected microbiomes mitigate chemical inhibition of plant performance. *New Phytol* 240:454–468. <https://doi.org/10.1111/nph.19249>
- Scavo A, Restuccia A, Mauromicale G (2018). Allelopathy: General principles and basic aspects for agroecosystem control. In: Gaba S, Smith B, Lichtfouse E (eds) *Sustainable Agriculture Reviews*, Springer: Cham, Switzerland, Volume 28, pp 47–101
- Scavo A, Mauromicale G (2021) Crop allelopathy for sustainable weed management in agroecosystems: knowing the present with a view to the future. *Agronomy* 11:2104. <https://doi.org/10.3390/agronomy1112104>
- Schrad N, Pensky J, Gorski G, Beganskas S, Fisher AT, Sal-tikov C (2022) Soil characteristics and redox properties of infiltrating water are determinants of microbial communities at managed aquifer recharge sites. *FEMS Microbiol Ecol* 98:fiac130. <https://doi.org/10.1093/femsec/fiac130>
- Sehgal A, Sita K, Bhandari K, Kumar S, Kumar J, Vara Prasad PV, Siddique KHM, Nayyar H (2019) Influence of drought and heat stress, applied independently or in combination during seed development, on qualitative and quantitative aspects of seeds of lentil (*Lens culinaris* Medikus) genotypes, differing in drought sensitivity. *Plant Cell Environ* 42:198–211. <https://doi.org/10.1111/pce.13328>
- Shi S, Richardson AE, O'Callaghan M, DeAngelis KM, Jones EE, Stewart A, Firestone MK, Condron LM (2011) Effects of selected root exudate components on soil bacterial communities. *FEMS Microbiol Ecol* 77:600–610. <https://doi.org/10.1111/j.1574-6941.2011.01150.x>
- St. Luce M, Grant C, Zebarth B, Ziadi N, O'Donovan J, Blackshaw R, Harker K, Johnson E, Gan Y, Lafond G, May W, Khakbazan M, Smith E (2015) Legumes can reduce economic optimum nitrogen rates and increase yields in a wheat–canola cropping sequence in western Canada. *Field Crops Res*. <https://doi.org/10.1016/j.fcr.2015.04.003>
- Stagnari F, Maggio A, Galieni A, Pisante M (2017) Multiple benefits of legumes for agriculture sustainability: an overview. *Chem Biol Technol Agric* 4:2. <https://doi.org/10.1186/s40538-016-0085-1>
- Taye ZM, Noble K, Siciliano SD, Helgason BL, Lamb EG (2022) Root growth dynamics, dominant rhizosphere bacteria, and correlation between dominant bacterial genera and root traits through *Brassica napus* development. *Plant Soil* 473:441–456. <https://doi.org/10.1007/s11104-022-05296-6>
- Thilakarathna MS, McElroy MS, Chapagain T, Papadopoulos YA, Raizada MN (2016) Belowground nitrogen transfer from legumes to non-legumes under managed herbaceous cropping systems. A review. *Agron Sustain Dev* 36:58. <https://doi.org/10.1007/s13593-016-0396-4>
- Tudi M, Daniel Ruan H, Wang L, Lyu J, Sadler R, Connell D, Chu C, Phung DT (2021) Agriculture development, pesticide application and its impact on the environment. *Int*

- J Environ Res Public Health 18:1112. <https://doi.org/10.3390/ijerph18031112>
- USDA FAS (2018) Oilseeds: world markets and trade (Foreign Agricultural Service/USDA). Available from: <https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf>
- Verret V, Gardarin A, Makowski D, Lorin M, Cadoux S, Butier A, Valantin-Morison M (2017) Assessment of the benefits of frost-sensitive companion plants in winter rapeseed. Eur J Agron 91:93–103. <https://doi.org/10.1016/j.eja.2017.09.006>
- Wang F, Jin F, Lin X, Jia F, Song K, Liang J, Zhang J, Zhang J (2024) *Priestia aryabhattai* improves soil environment and promotes alfalfa growth by enhancing rhizosphere microbial carbon sequestration capacity under greenhouse conditions. Curr Microbiol 81:420. <https://doi.org/10.1007/s00284-024-03946-9>
- Wolińska A, Kuźniar A, Zielenkiewicz U, Izak D, Szafranek-Nakonieczna A, Banach A, Błaszczyk M (2017) *Bacteroidetes* as a sensitive biological indicator of agricultural soil usage revealed by a culture-independent approach. Appl Soil Ecol 119:128–137. <https://doi.org/10.1016/j.apsoil.2017.06.009>
- Xia M, Ma X, Liu J, Wu M, Li Z, Liu M (2023) Potential effect of key soil bacterial taxa on the increase of rice yield under milk vetch rotation. Front Microbiol. <https://doi.org/10.3389/fmicb.2023.1150505>
- Xuan TD, Tsuzuki E (2002) Varietal differences in allelopathic potential of alfalfa. J Agron Crop Sci 188:2–7. <https://doi.org/10.1046/j.1439-037x.2002.00515.x>
- Xue Y, Xia H, Christie P, Zhang Z, Li L, Tang C (2016) Crop acquisition of phosphorus, iron and zinc from soil in cereal/legume intercropping systems: a critical review. Ann Bot 117:363–377. <https://doi.org/10.1093/aob/mcv182>
- Xue DY, Chen WF, Yang GP, Li YG, Zhang JJ (2025) Screening and application of highly efficient rhizobia for leguminous green manure *Astragalus sinicus* in lyophilized inoculants and seed coating. Plants 14(15):2431. <https://doi.org/10.3390/plants14152431>
- Yan G, Xing Y, Xu L, Wang J, Dong X, Shan W, Guo L, Wang Q (2017) Effects of different nitrogen additions on soil microbial communities in different seasons in a boreal forest. Ecosphere. <https://doi.org/10.1002/ECS2.1879>
- Yan E, Munier-Jolain N, Martin P, Carozzi M (2024) Intercropping on French farms: reducing pesticide and N fertiliser use while maintaining gross margins. Eur J Agron 152:127036. <https://doi.org/10.1016/j.eja.2023.127036>
- Yang L, Dai J, Gu C, Li Y, Hu W, Gao Y, Zhang Y, Liao X, Qin L (2025) Enrichment of root-associated bacterial communities by root metabolite profiles influences oilseed rape tolerance to nitrogen deprivation. Plant Stress 16:100827. <https://doi.org/10.1016/j.stress.2025.100827>
- Yazaki W, Shimasaki T, Aoki Y, Masuda S, Shibata A, Suda W, Shirasu K, Yazaki K, Sugiyama A (2021) Nitrogen deficiency-induced bacterial community shifts in soybean roots. Microb Environ 36:n/a. <https://doi.org/10.1264/jsme2.ME21004>
- Yu Z, Liu J, Li Y, Jin J, Liu X, Wang G (2018) Impact of land use, fertilization and seasonal variation on the abundance and diversity of *nirS*-type denitrifying bacterial communities in a mollisol in northeast China. Eur J Soil Biol 85:4–11. <https://doi.org/10.1016/j.ejsobi.2017.12.001>
- Zampieri E, Sillo F, Metelli G, Cucu MA, Montesano V, Quagliata G, Philipp L, Brescia F, Conte A, Giovannini L, Mennone C, Fiore A, Astolfi S, Savatin D, Sestili F, Reitz T, Balestrini R (2025) Insights into the influence of inter-cropping and arbuscular mycorrhizal inoculation on two modern durum wheat cultivars and their associated microbiota. Biol Fertil Soils 61:85–107. <https://doi.org/10.1007/s00374-024-01872-3>
- Zeng J, Liu X, Song L, Lin X, Zhang H, Shen C, Chu H (2016) Nitrogen fertilization directly affects soil bacterial diversity and indirectly affects bacterial community composition. Soil Biol Biochem 92:41–49. <https://doi.org/10.1016/J.SOIL-BIO.2015.09.018>
- Zhang P, Li B, Wu J, Hu S (2019) Invasive plants differentially affect soil biota through litter and rhizosphere pathways: a meta-analysis. Ecol Lett 22:200–210. <https://doi.org/10.1111/ele.13181>
- Zheng X, Koopmann B, Ulber B, von Tiedemann A (2020) A global survey on diseases and pests in oilseed rape—current challenges and innovative strategies of control. Front Agron 2:590908. <https://doi.org/10.3389/fagro.2020.590908>
- Zhou Q, Chen J, Xing Y, Xie X, Wang L (2019) Influence of intercropping Chinese milk vetch on the soil microbial community in rhizosphere of rape. Plant Soil. <https://doi.org/10.1007/s11104-019-04040-x>
- Zhou M, Sun C, Dai B, He Y, Zhong J (2023) Intercropping system modulated soil-microbe interactions that enhanced the growth and quality of fluecured tobacco by improving rhizospheric soil nutrients, microbial structure, and enzymatic activities. Front Plant Sci 14:1233464. <https://doi.org/10.3389/fpls.2023.1233464>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.

Chapter IV

This chapter is dedicated to a work published on Discover Soil in 2024. The experiment consisted in a mesocosm trial carried out using de-sealed soil of 2023 from a sealed urban site in Prato, Tuscany (43° 52' 09.1 " N 11° 03' 54.6" E), in the frame of the project Prato Urban Jungle (<https://www.uia-initiative.eu/en/uia-cities/prato>).

The experimental design consisted in four different conditions: *Trifolium repens* pots, *Cynara cardunculus* pots, *C. cardunculus* inoculated with *Funneliformis mosseae* and *Rhizophagus irregularis* and control pots with only the de-sealed soil. The objectives of the work were to understand if an AM fungal inoculum can colonize plants in a de-sealed soil, if the inoculum and the cover plants can restore soil health and if the native AMF can survive, recover and colonize plant roots after soil de-sealing. In particular, the soil status was assessed *via* volatile organic compounds (VOCs) screening and metabarcoding of the soil microbial communities to identify putative taxa associated with VOCs degradation or production.

This work demonstrates that AMF are able to colonize plant roots in de-sealed soil and that cover plants associated with AM fungal inoculation restore soil health status improving soil bacterial diversity. Several soil bacteria putatively involved in the degradation of anthropogenic VOCs derived from pollutants, as well as bacteria involved in the synthesis of metabolites able to improve soil nutrients availability were detected, underlining the importance of this combined method in restoring urban soil health status.

Microbial diversity and cover plants in de-sealed urban soil as strategies for mitigating anthropogenic volatile organic compounds

Maria Alexandra Cucu¹ • Luisa Neri² • Fabiano Sillo¹ • Elisa Zampieri¹ • Alice Calvo^{1,3} • Luca Giovannini¹ Cinzia De Benedictis² • Alessandro Zaldei² • Beniamino Gioli² • Rita Baraldi² • Raffaella Balestrini⁴ 

Published online: 02 December 2024

Received: 20 July 2024/ Accepted: 6 November 2024

The Author(s) 2024

[OPEN](#)

Abstract

Urbanization exacerbates soil degradation, compromising global soil health and biodiversity. To reduce this, strategies for soil de-sealing and bioremediation are necessary. In a mesocosm experiment using a de-sealed soil from an urban site as substrate, an inoculum of arbuscular mycorrhizal fungi (AMF) and two cover plants, *Cynara cardunculus* L. and *Trifolium repens* L., were utilized to assess their effects on volatile organic compound (VOC) emissions and soil microbial community dynamics. The soil status was investigated employing soil VOC and metabarcoding analyses. Our results showed that the soil VOC emission was mainly composed by anthropogenic derived VOCs, especially arene and furan classes. These two classes were shown to be less concentrated in *C. cardunculus* soils, with and without AM fungal inoculation, suggesting a positive impact of these conditions on soil health restoration. Additionally, AM fungal inoculation of *C. cardunculus* resulted in increased bacterial alpha diversity, with enhanced Proteobacteria/Acidobacteria, Cyanobacteria/Chloroflexi, and Fungi/Bacteria ratios, suggesting an improvement in soil quality conditions. In our experimental conditions, order Chloroflexales and specific bacterial genera, including *Ralstonia*, *Delftia*, *Ramlibacter*, were identified as contributors to VOC degradation, highlighting their adaptability in contaminated environments. Overall, this study provided evidence on the importance of integrating AM fungal inoculation and specific flowerbed/ornamental plants in urban soil management after de-sealing processes.

Keywords AMF- Beneficial microbes- Bioremediation- Urbanization- Pollutant breakdown- Soil degradation- Soil microbiome

Maria Alexandra Cucu and Luisa Neri contributed equally as first authors.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s44378-024-00022-3>.

@ Raffaella Balestrini, raffaellamaria.balestrini@cnr.it | ¹National Research Council, Institute for Sustainable Plant Protection, Strada delle Cacce 73, 10135 Turin, Italy. ²National Research Council, Institute of BioEconomy, Via P. Gobetti 101, 40129 Bologna and Via G. Caproni 8, 50145 Florence, Italy. ³Department of Agriculture and Desertification Research Centre, University of Sassari, Sassari, Italy. ⁴National Research Council, Institute of Biosciences and BioResources, Via G. Amendola 165/A, 70126 Bari, Italy.



Discover Soil

(2024) 1:22

<https://doi.org/10.1007/s44378-024-00022-3>

1 Introduction

Global urbanization exacerbates climate change [1, 2], causing extreme ecosystem alterations that impact soil health [3–5]. By 2050, it is predicted that half of the world's population will live in urban areas [6], leading to land degradation [7] and a decline in soil ecosystem services [6, 8, 9]. Soil sealing, which involves covering soil with waterproof materials due to urban development [10], is considered a form of land degradation that negatively impacts soil health [11]. This process can have a negative impact on soil health, by reducing its fertility, biodiversity, water infiltration, increasing surface runoff and pollution, and intensifying greenhouse gas emissions [12–14]. To mitigate these effects, there is an urgent need to increase green spaces in urban areas. The primary recommended actions include soil de-sealing, to reinstate the optimal soil physicochemical properties [15–16]. However, the use of de-sealed soils in urban green spaces is still challenging, since these soils can be potentially contaminated by anthropogenic compounds, as well as they may have issues with soil compaction and drainage [15]. Additionally, the potential fertility of soils that have been de-sealed remains largely unknown [15].

In this scenario, a promising approach to restore the health status of de-sealed soils can be represented by bioremediation [17]. Bioremediation is an eco-friendly method that involves the use of soil microbes and plants (i.e., phytoremediation), suitable to restore soil health in urban areas [17–18]. This approach has deemed to reduce soil pollutants also coming from urban activities such as traffic and industries [19–20]. The use of arbuscular mycorrhizal fungi (AMF) is considered an important tool for improving soil health and facilitating ecological restoration [21, 22]. Particularly, AMF form symbiotic associations with approximately 80% of land plants, enhancing soil fertility by increasing plant nutrient uptake and promoting their growth [23, 24]. They are crucial for plant survival, especially in polluted environments, as they can enhance plant tolerance through improved mineral nutrition [25]. AM fungal mycelium may help to compensate for reduced root growth caused by pollutants, and some fungi are well-adapted to harsh soil conditions [26]. The Mycorrhizal Assisted Remediation (MAR) effectively remediated soils contaminated with organic and inorganic pollutants, such as heavy metals and poly-aromatic hydrocarbons (PHAs) [27–31]. Additionally, the use of both AM fungal and bacterial inocula (e.g., *Bacillus*, *Sphingomonas*, *Acinetobacter* sp.), or of inocula based on *Trichoderma* sp., has allowed to reduce soil contamination. [32–36]. However, to the best of our knowledge, the potential of AM fungal inoculation in restoring de-sealed soils has not yet been explored. Although colonization can still begin from resting spores, hyphae, or root fragments containing fungal structures, even in highly hydrocarbon-polluted sites [25], no information is available regarding the survival and functionality of native AMF inhabiting degraded de-sealed soils. Concerning phytoremediation, cardoon (*Cynara cardunculus* L.; [37–39]) and white clover [*Trifolium repens* L.; [40, 41)] are promising plant species in Mediterranean and temperate areas, respectively. They are considered for their ability to thrive in stressful, polluted environments and can absorb and accumulate contaminants, contributing to sustainable soil remediation and ecosystem restoration [42, 43].

Soil microbial communities are intrinsically linked to its physicochemical properties, and are adapted to its various environmental conditions [44–48]. In addition, the microorganisms are also able to degrade toxic compounds [49–55], playing a crucial role in restoring soil health by enhancing pollutant breakdown [48]. Changes regarding the improvement of soil health status may be predictable based on several indicators [56–59] and volatile organic compound (VOC) profiling is still a pioneer method [60, 61]. Soils represent both a sink and a source of VOCs [62–64]. Along with the presence of biogenic VOC (BVOC) imprint (e.g., alcohols, ketones, and terpenes) the de-sealed soils may have a strong anthropogenic VOC (AVOC) signature given by BTEX (benzene, toluene, xylene, and related chemicals) and polycyclic aromatic hydrocarbons (PAHs) with negative effects on human health [65, 66]. Nevertheless, reusing the soil after de-sealing may promote a fast breakdown of these VOCs from both biological and chemical point of view [67, 68]. Hence, the VOC profile could mirror the soil health status and could enhance our knowledge regarding the microbial community activity in soils subjected to different managements and bioremediation methods.

The aim of this study was to investigate the status of an urban soil after de-sealing using VOC emission and microbial communities as markers for soil health and fertility in a mesocosm experiment with soil collected from a recently de-sealed area. Particularly, a bioremediation approach based on the use of *Cynara cardunculus* and *Trifolium repens* as flowerbed/ornamental, due to the important role of these species in phytoremediation and their utilization in urban greenspaces was exploited. In addition, *C. cardunculus* pots were also inoculated with a commercial AM fungal inoculum, to assess the combined effect mediated by both plants and beneficial fungi. The rationale behind this study was connected to three questions:

- 1 can an AM fungal inoculum successfully colonize plants in a reused soil after de-sealing?
- 2 can an AM fungal inoculum and cover plants be effective in restoring the health status of a de-sealed soil through the improvement of pollutant (i.e., AVOC) breakdown?
- 3 can native AMF survive, recover and colonize plant roots after soil de-sealing, further contributing to the soil health status restoration?

2 Materials and methods

2.1 Site and soil sample collection

The soil sampled in 2023 has originated from a sealed urban site in Prato, Tuscany (43° 52' 09.1" N 11° 03' 54.6" E), in the frame of the project Prato Urban Jungle (<https://www.uia-initiative.eu/en/uia-cities/prato>). The project aimed to re-naturalize areas of the Prato Municipality by improving the soil through de-sealing and further developing "Urban Jungles", i.e., regions with a high density of green. After de-sealing intervention, approximately 40 kg of soil was collected from different 18 random points at approximately 60 cm depth below the road surface, and stored at 4 °C. The sampled site showed a complex subsurface composition, which included the following layers: the uppermost layer made up of asphalt at a depth of 10–15 cm, and beneath the asphalt layer at a depth ranging from 30 to 50 cm, the original gravel subbase was present. The de-sealed soil averaged a sandy loam texture (80% sand, 12% silt, 8% clay), very low organic matter (0.5%) and total nitrogen (0.4%) content, and moderately alkaline pH (7.8). The physical and chemical characteristics of the soil immediately after de-sealing (TOO are reported in Table 1.

2.2 Experimental design and sampling

A mesocosm experiment was carried out using the sampled de-sealed soil. The experiment was performed in 5 L capacity pots, filled with 66% of sampled soil and 33% of sterile sand. The experimental design included three conditions with three replicates each, as follows: *Trifolium*—pots where seeds of *Trifolium repens* were sown, *Cynara*—pots sown with *C. cardunculus*, *Cynara* MYC—pots sown with *C. cardunculus* in which, prior sowing, the soil was mixed with a mixture of two commercial AM fungal inocula (INOQ, Germany), composed by *Funneliformis mosseae* and *Rhizophagus irregularis* (1:1). In addition to these three conditions, a Ctrl—control soil neither inoculated nor sown—was also considered. Pots were prepared at the end of March 2023, watered with 1 L of tap water for each pot every two days, and maintained in an open space under natural climatic conditions [average temperature between 17–35 °C, average precipitation between 53 and 155 mm (<https://www.arpa.piemonte.it/> and <https://www.weatherworld.com/yearly-climate/it/turin.html>)] for six months, until the end of September 2023. At this date (T1), a visual inspection of spontaneous flora grown during the experimental time course was performed (supplementary information SI). Immediately after plants removal, the soil sampling has been performed for all the pots. After homogenization, one part of the soil samples was air-dried, 0.2 mm sieved and used for the physical and chemical characterization according to standardized protocols by an analytical laboratory (Demetra, Pescia, Italy). Two additional parts of the sampled soil were kept at –20 °C for metabarcoding or at 4 °C for VOC measurements, carried out within a week from collection.

2.3 Assessment of arbuscular mycorrhizal (AM) fungal colonization

Arbuscular mycorrhizal fungal colonization was assessed on all plant roots from both inoculated and non-inoculated (i.e., control) pots, including *C. cardunculus*, *T. repens* and all spontaneous grown plant species, considering each pot as a biological replicate ($n = 3$). Roots were cleared with a KOH (10%) solution in a water bath at 80 °C for 30 min, then stained at room temperature overnight with 0.1% Cotton Blue in lactic acid. The next day, the roots were washed several times with lactic acid and the percentage of arbuscules, vesicles and hyphae was estimated under a dissecting microscope using the grid line intersection method [69].

2.4 Assessment of soil microbial biodiversity through metabarcoding

Total genomic DNA was extracted from approximately 400 mg of each soil sample using the DNeasy PowerSoil Pro Kit (Qiagen GmbH, Hilden, Germany), following the manual instructions, for a total of 12 samples. Prior sequencing

Table 1 Soil physical—chemical parameters at TO and T1 in all three considered conditions: *Cynara*, *Trifolium* and *Cynara* MYC as well as in Ctrl soil samples

Conditions	pH	CE	Salinity	Nt	Pass	K	Ca	Mg	Fe	Mn
		mS/cm	%o	N g/Kg	P ₂ O ₅ /mg/Kg	K ₂ O mg/Kg	Ca mg/Kg	Mg mg/Kg	Fe mg/Kg	Mn
TO	7.7	0.15	0.2	0.4	29	43	1169	55	14	6
T1-Ctrl	7.8	0.19	0.2	0.3	25	28	772	30	14	3
T1-Cynara	8.1	0.16	0.2	0.2	17	16	624	23	9	6
T1-Trifolium	8	0.17	0.2	0.3	15	24	541	28	8	4
T1-Cynara MYC	8.2	0.14	0.2	0.2	18	26	543	25	8	3

“CE electrical conductivity, Nt total nitrogen, Pass assimilable phosphorus, K potassium, Ca calcium, Fe iron, Mn manganese, Cu copper, Zn zinc, B boron, SOM soil organic matter, C/N carbon/nitrogen ratio

(Novogene), DNA quantity and purity were measured using a Nanodrop 2000 (Thermo Scientific, Wilmington, U.S.A.). The bacterial 16S rRNA gene V3-V4 hypervariable regions and the fungal nuclear ribosomal ITS2-5.8S regions were sequenced. Sequencing libraries were generated using TruSeq™ DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. Library quantity and quality was assessed using the Qubit® 2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated. Raw sequence files were submitted to the NCBI Sequence Read Archive (SRA) under the Bioproject ID PRJNA1133247.

The 16S and ITS sequences were clustered into amplicon sequence variants—ASVs [70] using QIIME 2 v2022.8 [71]. The taxonomic annotation was performed using the Silva database version 138 [72] and the UNITE database [73] for 16S and ITS data, respectively. Additionally, the MaarjAM database [74] was used for the AMF to pre-train the Naive Bayes classifier sequences (99% identity) for AM fungal taxonomic assignment of ITS sequences. The ASV tables were used as inputs for Microbiome Analyst 2.0 (<https://www.microbiomeanalyst.ca/>; Chong et al. [75]) for data visualization and statistical assessment. Data were filtered to remove low quality and not informative features by setting the minimum count of the low counter filter at two and the prevalence in samples at 10%. Data were then rarefied to the minimum library size, scaled with the total sum scaling method.

2.5 Volatile organic compound (VOC) analysis

Within a week after soil sampling, emitted VOCs have been determined as described in Neri et al. [60] and in Sillo et al. [61], for all the three conditions [*Trifolium*, *Cynara*, and *Cynara* MYC] and the Ctrl soil samples. In detail, before VOC analysis, 12 soil samples were homogenized and stabilized at ambient temperature. Volatile emission was measured from 300 g soil using a dynamic headspace (DHS) technique continuously flushing VOC-free air obtained through a custom-made catalytic converter (ZAG) to purify air from undesired VOC and contaminant, collecting the soil enriched air exiting the DHS in specific traps filled with 200 mg of Tenax and Carbograph at a flow rate of 200 ml min⁻¹. Traps were desorbed using a thermal desorber (Markes International, Series 2 Unity) and analyzed with a 7890A gas chromatograph coupled with a 5975C mass detector (GC—MS, Agilent Technologies), following the method of Baraldi et al. [76]. Identification was carried out by comparing retention times and fragmentation patterns with the NIST 11 database and external reference compounds. The identified compounds, after correcting for VOC deriving from sampling system, were quantified using external standard calibration. VOC emissions were expressed as ng kg h.

2.6 Statistical analysis

Statistical assessment of diversity at microbial level was performed through the estimation of different ecological indices. Concerning microbial diversity in soil, both communities, total bacteria and fungi, and AMF were characterized according to alpha-diversity indices (Chao1 and Shannon indices) within samples. Additionally, beta-diversity among conditions was assessed by PERMANOVA using the Bray—Curtis dissimilarity index and Principal Coordinate Analysis (PCoA) for result visualization. Statistical differences among bacterial and fungal communities were assessed using univariate test in Microbiome Analyst 2.0. Furthermore, based on the prokaryotic diversity results, the annotation tool FAPROTAX [77] has been used to ascribe the functional ecological role to the identified bacteria.

For bacteria, the ratios between Proteobacteria (copiotrophs) and Acidobacteria (oligotrophs) (P/A) that is indicative of the trophic level of the soil [78], was calculated. In addition, Cyanobacteria/Chloroflexi (C/C) ratio, which was proposed as an early warning tool of soil degradation [79], was estimated. The Fungi/Bacteria (F/B) ratio was also considered, since higher abundance ratio of fungi to bacteria may suggest higher degree of ecosystems sustainability [80, 81]. Differences in the calculated ratios were statistically assessed by Student's t test ($p < 0.05$), using Ctrl as reference.

To assess the presence of putative positive/negative correlations between specific microbial taxa and VOC class amounts, a Spearman rank correlation co-occurrence network analysis at the order level was conducted with a p-value cutoff of 0.05 and a correlation threshold of 0.5 using R version 4.2.1. To test the influence of soil chemical properties and VOCs in all considered conditions, including Ctrl, a Principal Component Analysis (PCA) was performed using the “made4” package of R. For positive correlations, the database mVOC 3.0 [82] was used to investigate the reported putative association between detected microbial taxa and emitted VOCs. Differences in emission rates of VOC categories and classes of each conditions [*Trifolium*, *Cynara*, and *Cynara* MYC] with respect to the Ctrl were assessed by one-way ANOVA followed by Tukey's HSD post hoc test (by using the software JMP v.7) and by Student's t test ($p < 0.05$).

3 Results

3.1 Soil physical-chemical parameters at T1

The physical-chemical characteristics of soil samples at the end of experimental time (T1) are reported in Table 1. Overall, with respect to the TO samples, a slightly increase of pH, C/N ratio and Zn content, along with a decrease in total N, soil organic matter (SOM), assimilable P, K, Ca, Mg, Fe, Cu and B content have been observed in the three tested conditions, i.e., *Trifolium*, *Cynara*, and *Cynara* MYC. Conversely, the pH was similar and the Fe content did not change in Ctrl soil samples at T1 with respect to TO (Table 1). The PCA based on physical—chemical soil parameters evidenced a clear separation of all three conditions from Ctrl, especially guided by pH and C/N ratio (Fig. S1a).

3.2 Arbuscular mycorrhizal (AM) fungal colonization

The grid method allowed to evaluate the AM fungal colonization assessment. Accordingly, a mean colonization level of 53% (range 19—72%) was observed in roots of all *C. cardunculus* plants collected from pots of *Cynara* MYC condition. Details about colonization percentage of all plants from pots of all considered experimental conditions were presented in Table 2. Typical AM fungal structures (i.e., intraradical hyphae, arbuscules, and vesicles) were observed within the root tissues of *C. cardunculus* plants from *Cynara* MYC pots (Fig. 1), while no typical structures associated to AMF were observed neither in roots from both *Cynara* and *Trifolium* conditions nor in those of Ctrl.

3.3 Composition and structure of soil microbial communities

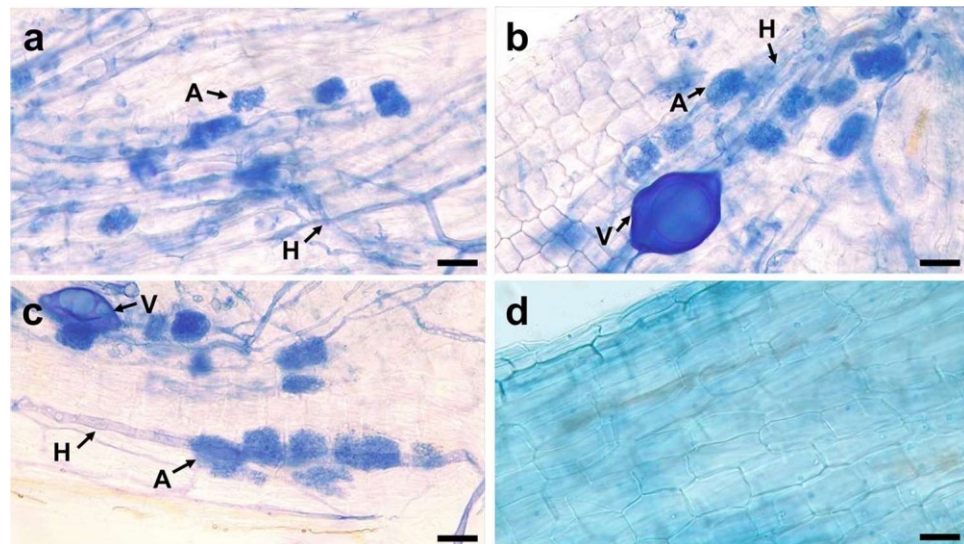
In profiling the microbial sequencing data, the high-quality sequences, obtained after quality filtering, were accurately mapped into bacterial and fungal ASVs, with a total of 387,698 total reads—35,254 average counts *per* sample for 16S, and 636,856 total reads—45,489 average counts *per* sample for ITS, respectively.

Table 2 Percentage* of mycorrhizal colonization in roots of grown plants in *Trifolium*, *Cynara*, and in *Cynara* MYC conditions

Condition	Plant species	Mycorrhizal colonization (t/t)
Ctrl	<i>Populus x canadensis</i>	
	<i>Euphorbia maculata</i>	
	<i>Salix aurita</i>	—
	<i>Ambrosia</i> sp.	—
	<i>Hypochaeris</i> sp.	—
<i>Trifolium</i>	<i>Trifolium repens</i>	—
	<i>Hypochaeris</i> sp.	—
	<i>Euphorbia maculata</i>	—
<i>Cynara</i>	<i>Cynara cardunculus</i>	—
	<i>Euphorbia maculata</i>	—
	<i>Populus x canadensis</i>	—
	<i>Salix aurita</i>	—
	<i>Salix triandra</i>	—
<i>Cynara</i> MYC	<i>Cynara cardunculus</i>	—
	<i>Populus x canescens</i>	—
	<i>Salix triandra</i>	19–72
	<i>Euphorbia maculata</i>	11
	<i>Ambrosia</i> sp.	0–47
	<i>Populus x canadensis</i>	60
		67
		48–72

A control soil neither inoculated nor sown (Ctrl) was also considered. Results are presented as range of percentage within plant species of each experimental condition

Fig. 1 Evaluation of mycorrhizal colonization by staining with cotton blue of *Cynara cardunculus* roots sown in pots inoculated with AMF [*Cynara* MYC], showing typical AM fungal structures, such as intraradical hyphae (H), arbuscules (A) and vesicles (V) within the root tissues (a—c); roots of *Cynara cardunculus* sown in the absence of AM fungal inoculum [*Cynara* did not show typical AM fungal structures (d). Bar= 30 μ m



Regarding the prokaryotic community, at Phylum level all soils were dominated by Proteobacteria with the highest percentage in *Cynara* soils (47%), followed by Ctrl (45%), *Cynara* MYC (37%) and *Trifolium* (33%); Acidobacteria with an average of 10% among these and 20% in the Ctrl; Actinobacteria in a percentage of 19% in *Trifolium* soil samples, followed by 15% in *Cynara* MYC soil samples and 12% in Ctrl and *Cynara* soil samples. Bacteroidota was among the dominant phyla in all conditions (5%) but not in the Ctrl. Chloroflexi phylum was dominant in *Trifolium* and *Cynara* MYC soil samples and Firmicutes was among the dominant phyla only in Ctrl soil samples. Cyanobacteria phylum was more abundant in *Cynara* MYC soil samples (3.7%), followed by *Cynara* (1.4%) and *Trifolium* (0.45%), whereas no representatives of this phylum were detected in Ctrl soil samples (Fig. 2a—d).

The most relevant Orders within Phylum/Class of each considered conditions were as follows: in Ctrl soil samples: Burkholderiales (Betaproteobacteria) (35%), Pseudomonadales (Proteobacteria) (7%), Gaiellales (Actinobacteria) (4%), Propionibacteriales (Actinobacteria) (3%); in *Cynara* MYC soil samples: Burkholderiales (Betaproteobacteria) (16%), Rhizobiales (Proteobacteria) (5%), Sphingomonadales (Alphaproteobacteria) (5%), Rhodobacterales (Alphaproteobacteria) (4%), Gemmatimonadales (Gemmatimonadota) (3%), Gaiellales (Actinobacteria) (3%); in *Cynara* soil samples: Burkholderiales (Betaproteobacteria) (27%), Vicinamibacteriales (Acidobacteria) (4%), Rhizobiales (Alphaproteobacteria) (4%); in *Trifolium*: Burkholderiales (Betaproteobacteria) (12%), Rhizobiales (Alphaproteobacteria) (7%), Vicinamibacteriales (Acidobacteria) (5%), Sphingomonales (Pseudomonadota) (4%) and Gaiellales (Actinobacteria) (3%) (Fig. 3).

The univariate test allowed the discrimination of several orders more abundant in soil samples of the three conditions compared to Ctrl (Table S1). In addition, the following genera were found more abundant with respect to the Ctrl: *Nocardioides* (Actinobacteria) (p-value 0.004, FDR 0.045) and *Sphingobium* (Proteobacteria) (p-value 0.005, FDR 0.05702) in *Cynara* soil samples, *Ramlibacter* (Pseudomonadota) (p-value 2.50E-04, FDR 0.034) in *Cynara* MYC soil samples and *Ralstonia* (Proteobacteria) (p-value 2.99E-05, FDR 0.006) in *Trifolium* soil samples.

In general, several genera/families were identified as core microbiome in all tested conditions as well as in the Ctrl, and they are reported in Table S2. Particularly, common genera included *Ralstonia*, *Sphingomonas*, *Gaiella*, *Rubellimicrobium*, *Haliangium*, *Ramlibacter*, *Pseudarthrobacter*, *Nocardioides*, *Latescibacter*, *Bacillus*, *Streptomyces*, *Bryobacter*, *Noviherbaspirillum*, *Novosphingobium*, *Nitrospira*, *Nocardia*, and *Pseudomonas*, and families included Vicinamibacteraceae and Vermiphilaceae. On the other hand, exclusive genera in Ctrl were *Caulobacter*, *Cupriavidus*, *Acinetobacter*, *Acidovorax*, *Massilia*, *Rhodococcus* (prevalence=0.33); in *Trifolium* soil samples: *Terrimonas*, *Microbacterium* (prevalence=0.33); in *Cynara* MYC soil samples: *Polycyclovorans*, *Paenibacillus* (prevalence=0.5); in *Cynara* soil samples: *Kapabacteriales* (prevalence=0.33).

Alpha-diversity of bacterial communities showed significant differences among samples (Chao 1 index, p-value 0.014). Accordingly, a higher diversity in *Trifolium* soil samples was found when compared with Ctrl (p-value 0.017362, FDR 0.104; Fig. 4a), followed by *Cynara* MYC and *Cynara* soil samples. The result was also confirmed by the Shannon index, which takes into account both richness and evenness (p-value 1.105E-05, FDR 74.845; Fig. 4b). Based on the beta-diversity, a clear separation of all three conditions from the Ctrl was shown (Fig. S2). The prokaryotic communities were more similar in *Cynara* MYC and *Trifolium* soil samples than those from Ctrl and *Cynara* soil samples (R 0.066, p-value <0.003).

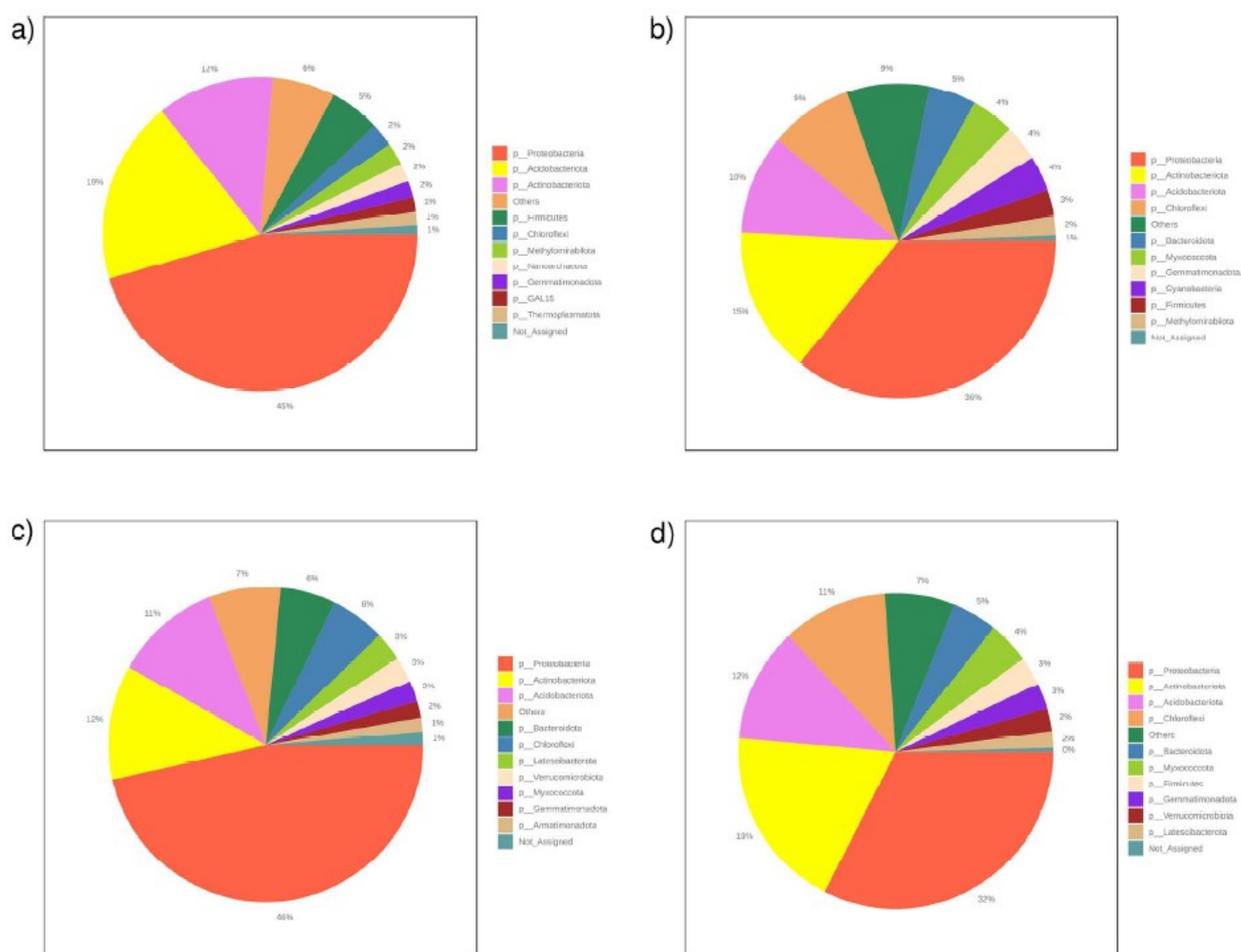
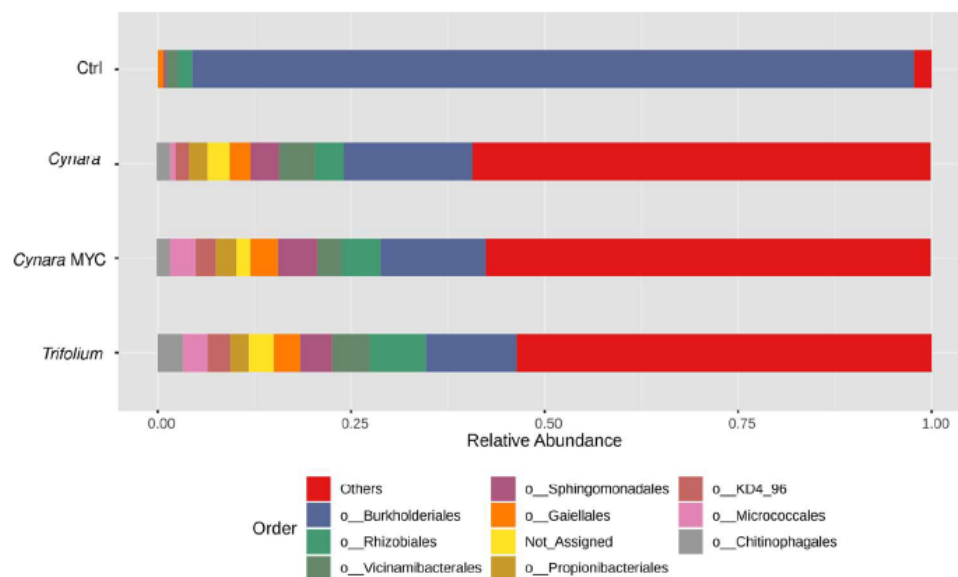


Fig. 2 Pie charts of main bacterial community composition at Phylum level in **a** Ctrl; **b** *Cynara* MYC; **c** *Cynara*, **d** *Trifolium* soil samples

Fig. 3 Stacked barplots of bacterial relative abundance of community composition at Order level in Ctrl, *Cynara* MYC, *Cynara* and *Trifolium* soils samples



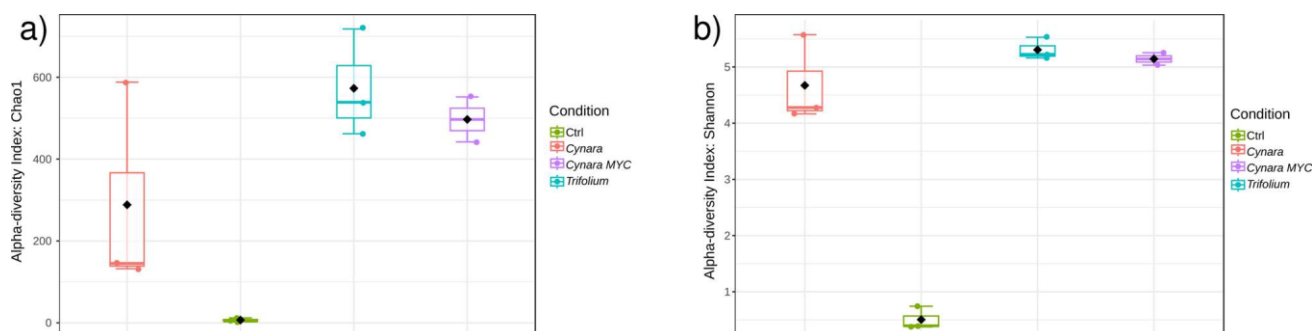


Fig. 4 Alpha diversity of bacterial communities in all three considered conditions: *Cynara*, *Trifolium* and *Cynara* MYC as well as in Ctrl soil samples: **a** Chao 1 index and **b** Shannon index

Concerning the fungal community, at Phylum level the soils were dominated by Mucoromycota (average 50%) and Ascomycota as follows: 23% in Ctrl, 19% in *Trifolium*, 16% in *Cynara* MYC inoculated soils and 5% in *Cynara* soil samples. However, an average percentage of 70% of reads was not assigned. At class level, Glomeromycetes was the most dominant one as follows: 49% in Ctrl soil samples, 43% in *Cynara* MYC soil samples, 31% in *Trifolium* soil samples and 11% in *Cynara* soil samples. The fungal orders were displayed in abundance pie charts, excluding the unclassified and unassigned ones (Fig. 5). From the assigned taxa, the most dominant fungal orders were identified as Sordariales (18%), Glomerales (16%), Capnodiales (14%) and Hypocreales (12%) in Ctrl (Fig. 5a); Hypocreales (57%), Pleosporales,

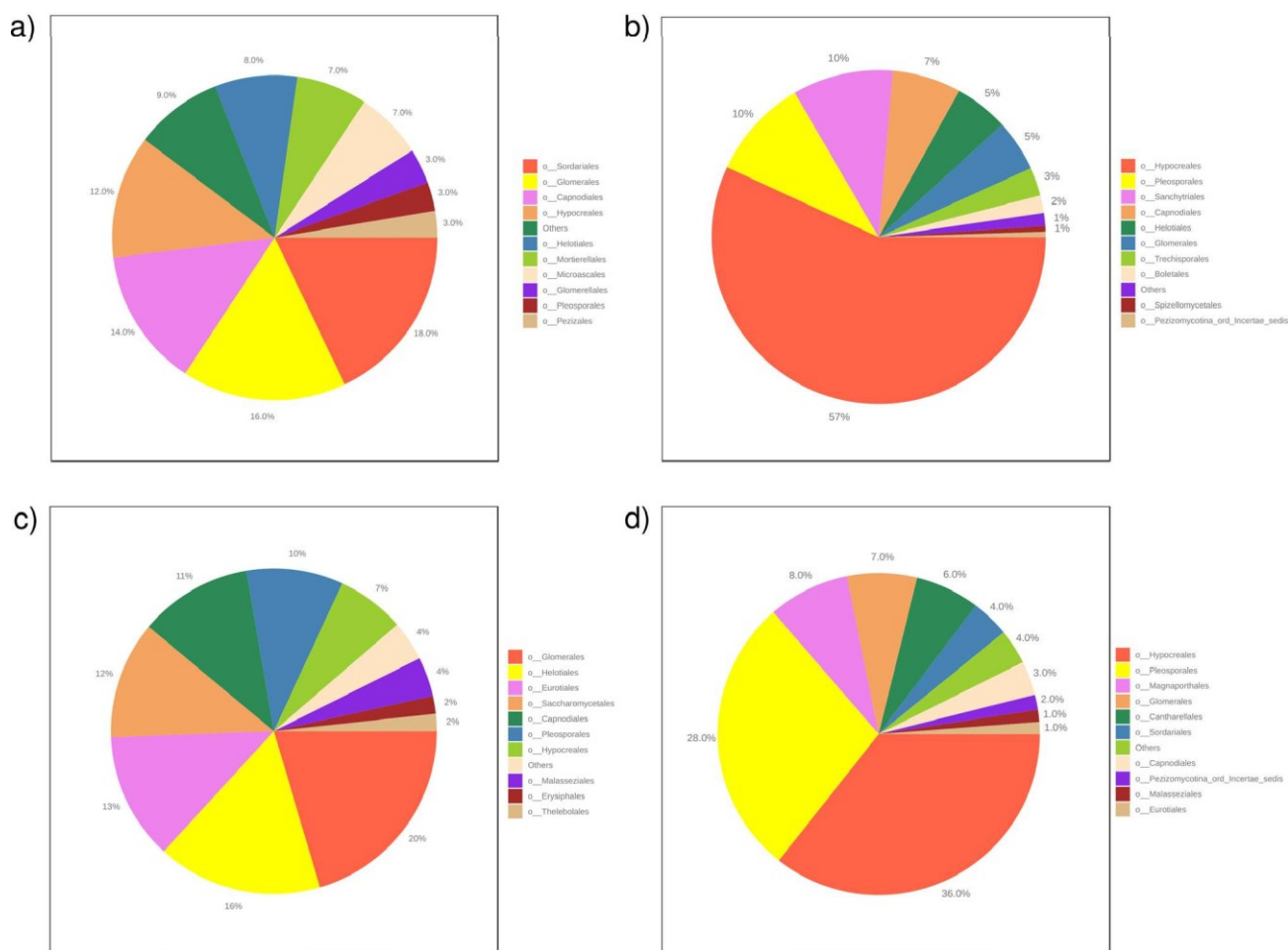


Fig. 5 Pie charts of main fungal community composition at Order level in **a** Ctrl; **b** *Cynara* MYC; **c** *Cynara*, **d** *Trifolium* soil samples

Sanchytriales (10%), Capnoidiales, Helotiales and Glomerales (7%) in *Cynara* MYC soil samples (Fig. 5b); Glomerales (20%) and Helotiales (16%), Eurotiales (15%), Saccharomycetales (12%), Capnoidiales (11%) in *Cynara* soil samples (Fig. 5c). The dominant fungi at order level in *Trifolium* soil samples were *Hypocreales* (36%), Pleosporales (28%), Magnaporthales (8%) and Glomerales (7%) (Fig. 5d).

Regarding the fungal core microbiome, the following genera were common to all conditions: *Cilomus*, *Hyalocypha*, *Cladosporium*, *Penicillium*, *Fusarium*. Based on univariate test, no significant differences in fungal taxa abundances were detected [$p > 0.05$].

Regarding the total fungal and AM fungal communities, no significative differences have been found on both alpha (Chao 1 index, p-value 0.522, FDR 0.811 and Shannon index, p-value 0.741, FDR 0.423) (Fig. 6a—d) and beta diversity (R^2 0.263, p-value 0.58). However, although not significant, a slight increase in alpha diversity of both total fungi and AM fungal communities has been observed in MYC soil samples (Fig. 6a—d).

Both Proteobacteria/Acidobacteria (P/A) and Cyanobacteria/Chloroflexi (C/C) ratios showed similar results, with significant higher values in *Cynara* (P/A=4.3; C/C=4) and *Cynara* MYC (P/A= 3.5; C/C=4) soil samples, with respect to *Trifolium* (P/A= 2.7; C/C=0.004) and Ctrl (P/A= 2.4; C/C=0) (Student's t-test, $p < 0.05$) (Fig. 7a). The Fungi/Bacteria (F/B) ratio showed a significant increase in *Cynara* MYC soil samples with respect to all other conditions [$p < 0.05$] (Fig. 7b).

Functional annotation of bacterial communities by FAPROTAX allowed to identify several categories reported in Fig. S3. There was a prominent occurrence of sequences of bacteria associated with putative aerobic chemoheterotrophy, with a higher abundance in *Trifolium* soil samples and a lower abundance in *Cynara* MYC soil samples. Aromatic hydrocarbon and aliphatic degradation showed higher abundances in *Trifolium* and Ctrl conditions. Denitrifies seemed to be more abundant in the *Cynara* soil samples, while methane processes were more prevalent in Ctrl soil samples. In addition, a high abundance of photoautotrophs was observed in *Cynara* MYC and *Cynara* soil samples with respect to the Ctrl soil samples.

3.4 Soil VOC signature

A total of 62 VOCs were identified in the soils samples from all the three experimental conditions [*Cynara*, *Trifolium* and *Cynara* MYC] and the Ctrl; the majority belonged to 14 chemical classes (acid, alcohol, aldehyde, alkane, alkene,

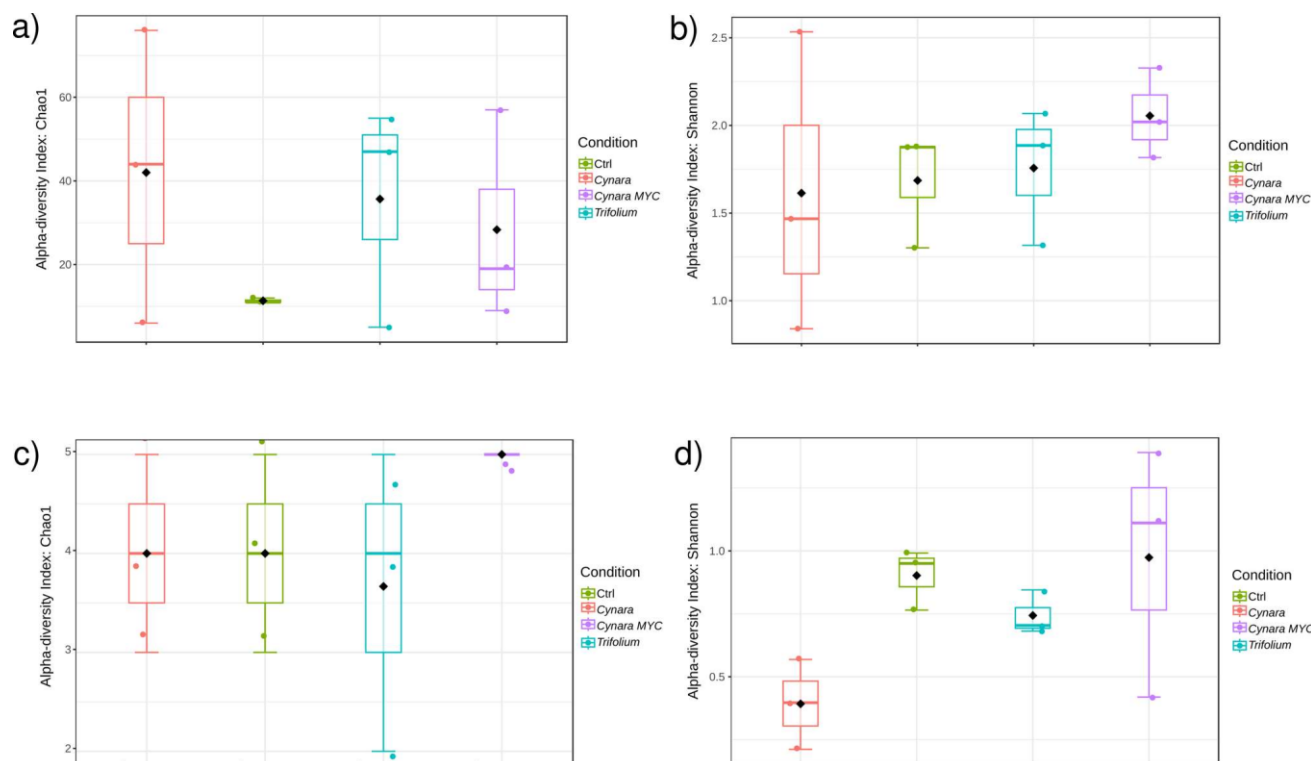


Fig. 6 Alpha diversity of total fungal communities **a** Chao 1 index and **b** Shannon index, as well as alpha diversity of AMF **c** Chao 1 index and **d** Shannon index, in all three considered conditions: *Cynara*, *Trifolium* and *Cynara* MYC as well as in Ctrl soil samples

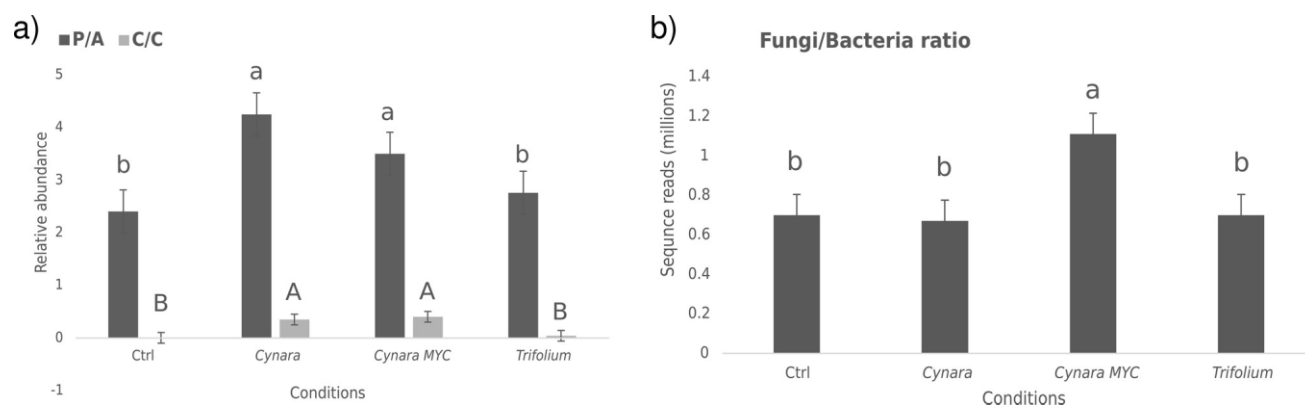


Fig. 7 Proteobacteria/Acidobacteria (P/A) and Cyanobacteria/Chloroflexi (C/C) ratios (a) and Fungi/Bacteria (F/B) ratio (b), in all three considered conditions: *Cynara*, *Trifolium* and *Cynara MYC* as well as in Ctrl soil samples (letters indicate significant differences at $p < 0.05$, based on Student's t test between the conditions and the control soil samples)

arene, ester, ether, furan, ketone, phenol, terpene, terpenoid, chloromethane), except for only two compounds belonging to other classes and one compound (putative n-C9) that could not be identified using the NIST 11 library (Table S3). Some significant differences in VOC emission among the experimental conditions were observed: the arene toluene was more abundant in Ctrl soil samples; the arene ethyl-benzene and p- and o-xylene were more abundant in *Trifolium* samples; the chloromethane dichloromethane was higher in Ctrl soil samples, the furan tetrahydrofuran and the organic disulfide dimethyl disulfide were more abundant in *Trifolium* soil samples (Table S3). Generally, the emitted VOC were mostly anthropogenic belonging mainly to furan and arene classes, which were significantly higher in the *Trifolium* soil samples (Fig. 8a). Considering VOC emission without the furan and arene classes, some significant differences were detected: the higher emission values have been observed for ester class, with significant differences among conditions and control as follows *Trifolium* — *Cynara* > *Cynara MYC* > Ctrl. No ether or very low concentration were observed in Ctrl and *Cynara* soil samples, respectively, while higher concentrations were detected in *Trifolium* and *Cynara MYC* soils. Low amounts of chloromethanes were detected in all of the treatments compared with the Ctrl. High emissions of acid compounds (acetic acid) were detected only in *Cynara MYC* soil samples and relatively high emissions, although not significant, of alcohols were observed in *Cynara MYC* and *Trifolium* soil samples (Fig. 8b).

As for soil chemical parameters, the PCA performed on all VOC classes showed distinct separation between the tested conditions and the Ctrl, driven mainly by terpene and ester classes (Fig. S1 b).

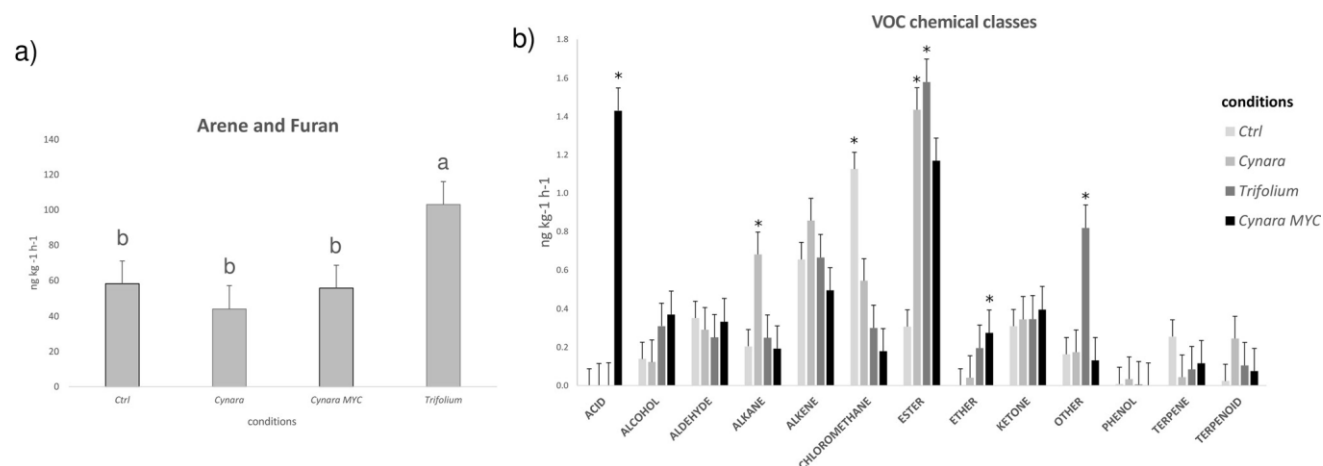


Fig. 8 Arene and Furan classes (a) and VOC chemical classes (b), in all three considered conditions: *Cynara*, *Trifolium* and *Cynara MYC* as well as in Ctrl soil samples (different letters and asterisks indicate significant differences at $p < 0.05$, based on Student's t test between the conditions and control soil samples)

3.5 Correlation between VOC emissions and microbial communities

Correlation between identified VOCs and microbial communities allowed to detect in general multiple bacterial and fungal taxa negatively associated to specific VOC classes, especially with those with anthropogenic origin. Outcomes from correlation between detected microbial orders and genus were reported in Tables S4—11. In *Cynara* MYC soil samples Chloroflexales as well as genera *Delftia*, *Ralstonia* and *Ramlibacter* were negatively correlated with arenes class. Ten orders have been found negatively correlated with esters class (Acidobacteriales, Chitinophagales, Kapabacteriales, Micromonosporales, Myxococcales, Pyrinomonadales, Reyranelles, Thermoactinomycetales, Vicinamibacteriales, Xanthomonadales), ten orders were found negatively correlated with alkenes class (Haliangiales, Kapabacteriales, Micromonosporales, Myxococcales, Oritales, Paenibacillales, Pyrinomonadales, Reyranelles, Thermoactinomycetales, Xanthomonadales). In *Cynara* MYC soil samples, also some positive correlations were detected: fifteen bacterial orders have been found positively correlated with acid class (i.e., acetic acid), with some important genera as *Lysinibacillus*, *Brevibacillus*, *Pseudomonas*, *Pseudoxanthomonas*, seven orders were found positively correlated with alcohol class (Acetobacteriales, Azospirillales, Chloroflexales, Cytophagales, Propionibacteriales, Sphingobacteriales, Sumerlaeales); four orders were found positively correlated with terpenoids class (Micrococcales, Microtrichales Sphingomonadales, Streptosporangiales). Additionally, positive correlations were observed with dimethyl sulfide at order (Kapabacteriales) and genus level (*Aeromicrobium*, *Ahniella*, *Haliangium*, *Luteobacter*, *Nitrospira*). In *Cynara* soil samples negative correlations dominated with phenol (Gaiellales, Holosporales, Methanomassiliicoccales), ketone (Myxococcota), furan (Coxiellales, Gemmatales, Pyrinomonadales, and *Coxiella*, *Flavobacterium*, *Paenibacillus* at genera level), ester (*Ramlibacter*), alkene (Methylomirabilota, Thermoplasmatota, Micrococcales, Rokubacteriales), alkanes (Desulfobacterota, Planctomycetota, Coxiellales, Gemmatales, Microtrichales, Pyrinomonadales), alcohol (Nitrospirota, Gemmatimonadales, Nitrospirales, Polyangiales, Thermoactinomycetales) and acid classes (*Ramlibacter*). Some positive correlations have been found for terpenoids (Microtrichales, Pedosphaerales, Solibacteriales) and terpene (i.e., limonene) (*Bauldia*) classes. In addition *Lacunisphaera*, *Sphingobium* genera were positively correlated with dimethyl disulfide. Negative correlations dominating the *Trifolium* soil samples have been found with 11 VOC classes, the predominant ones with furans: Nitrospirales, Polyangiales, Salinisphaerales, Sphingomonadales, Woeseearchaeales at order level and *Acinetobacter*, *Ammoniphilus*, *Brevibacillus*, *Coxiella*, *Dongia* at genus level. In *Trifolium* soil samples *Ammoniphilus* genus was positively correlated with dimethyl disulfide. In Ctrl soil samples, general negative correlations at phylum level were observed with furan class (Proteobacteria) and terpenes at order level (Vicinamibacteriales). Vicinamibacteraceae was also negatively correlated with dichloromethane compound. Positive correlation were found with terpenoids class (Vicinamibacteriales), and genus level with terpenes [*Nocardiodides*, *Cupriavidus*], phenol [*Actinoplanes*, *Devosia*, *Gaiella*, *Nocardia*, *Sphingomonas*], ester [*Caulobacter*, *Stenotrophomonas*] and alkene [*Actinoplanes*, *Devosia*, *Gaiella*, *Nocardia*, *Sphingomonas*] classes.

Regarding the fungal communities, negative correlations dominated in all conditions, with Trechisporales, Eurotiales *Penicilium* sp., Capnodiales and *Glomus* negatively correlated with furan, arene, alkene and alkane classes, respectively, in *Cynara* MYC soil samples; Pleosporales, Hypocreales, Eurotiales, Glomerales, Helotiales negatively correlated with terpenoid, furan, ester, arene and alcohol classes in *Trifolium* soil samples; Hyaloscypha, Capnodiales, Glomerales, Sordariales, Hypocreales negatively correlated with phenol, ketone and arene classes (Tables S8—S11).

Concerning the positive correlations, the mVOC3.0 database confirmed the microbial origin of acetic acid: *Bacillus* sp. [83], and *Pseudomonas* sp. [84, 85].

4 Discussion

Rapid urbanization is transforming our cities, reducing green spaces, which are essential for mitigating the effects of global warming and for enhancing urban biodiversity [19, 86, 87]. Soil de-sealing and further bioremediation management are crucial for renovating soil health, which is mainly defined by the stability and resilience of the soil microbiome [88].

4.1 Application of AM fungal inoculum might enhance microbial diversity and soil health status

Our results showed that a de-sealed soil could restore its health and fertility when specific cover plants (e.g., *C. cardunculus*), both alone or inoculated with AMF have been used. Renella [89] demonstrated “in situ” the recovery of soil fertility after de-sealing of urban soils that underwent colonization by spontaneous plants. To the best of our knowledge this was the first study, which evidenced a successful AM fungal colonization of plant roots from a de-sealed, degraded and reused soil, by a commercial AM fungal inoculum. On the other hand, no colonization was observed where the inoculum was not added, thus suggesting that despite the relative high abundance of Glomeromycetes in control soils, the native AMF lost their colonization potential, probably due to the contaminants and harsh conditions induced by soil sealing. Previous studies suggested that AM fungal spores can still colonized host roots effectively in degraded polluted soils (e.g., heavy metals), despite low counts [90,91] Cabello et al. [25] pointed out that native AM fungal propagules from extremely hydrocarbon polluted soils had a high colonization capacity. On the other hand, our findings aligned with broader observations indicating that soil degradation generally resulted in decreased diversity and colonization capacity of native AMF [92]. Further studies on how different materials used for soil sealing (e.g., semipervious pavements) as well as sealing duration may affect functionality of native AMF should be considered.

Both *C. cardunculus* with and without inoculum played a crucial role in enhancing soil bacterial diversity, confirming the “Microbiome Rewilding Hypothesis” [88]. The beneficial effects on soil fertility of *C. cardunculus* as cover plant, as well as the symbiotic relationships formed by AMF with plant roots, created favorable conditions for the establishment of diverse bacterial communities. This increased bacterial diversity may further have contributed to the overall soil status resilience and improvement. The importance of symbiotic interactions in promoting soil health and vegetation growth efficiency was emphasized, as shown by the estimation of the detected spontaneous plant species richness, when mycorrhizal inoculum and *C. cardunculus* were used (Fig. S4). The presence of *C. cardunculus* alone, as well as AMF and *C. cardunculus* consociation, positively influenced the soil health status, as indicated by shifts in key microbial community ratios. The diverse array of bacterial species was also underlined by the pH change, widely recognized indicator of functional microbial patterns [93, 94]. The increase in Proteobacteria/Acidobacteria ratio, along with the enhanced Cyanobacteria/Chloroflexi, suggested a favorable shift within copiotroph communities, as r-strategists, towards increased nutrient availability. These changes aligned with findings by Smit et al. [78], indicating a potential restoration of soil fertility and improvement in nutrient cycling processes. Moreover, Chloroflexi and Cyanobacteria could be used as tool of soil degradation status, as members of Chloroflexi phylum may have a competitive advantage over Cyanobacteria in nutrient-limiting environments [96] as observed in control soils and in pots where *Trifolium* was sown.

Noteworthy, the observed shift from bacterial to fungal dominance, as showed by the Fungi/Bacteria ratio, in soils of *C. cardunculus* plants colonized by AMF suggested a potential role of mycorrhizal fungi in the improvement of the health soil status [95]. This was further evidenced also by the higher diversity of total fungi as 4-strategists in inoculated soils, and by lower Ascomycota abundance in *C. cardunculus* pots with inoculum, which may represent an indicator of ecosystem health recovery [96].

4.2 The interaction between *C. cardunculus* and AMF contributed to AVOC biodegradation and restoration of soil health status

In our study multiple negative correlations between microbial communities and the identified compounds have been observed, supporting a clear VOC signature, which indicated an overall degradation/consumption pattern. This underlined the fact that soil uptake based on anthropogenic compound degradation may have exceeded the overall VOC production, as they were readily available, as inferred by Jiao et al. [97]. In our experiment, the degradation process was sustained by the soil core microbiome identified as an unique consortium containing genera able to degrade AVOCs (i.e., BTEX -benzene, toluene, ethylbenzene, xylene and chlorinated methane) under aerobic conditions. This soil core microbiome included taxa reported to be degraders of anthropogenic based VOC, such as *Pseudomonas*, *Cupriavidus* [98], *Streptomyces* [99], *Acinetobacter* [100], *Bacillus* [101], *Microbacterium* [102], *Massilia* [103], and *Rhodococcus* [104]. Particularly, bacterial strains such as *Acinetobacter* sp., *Pseudomonas* sp., and *Rhodococcus* sp. are well known to secrete biosurfactants to degrade oil-based contaminants [105].

Our FAPROTAX-based analysis highlighted the functional roles of chemoheterotrophs as r-strategists, in nutrient cycling within de-sealed and reused soils. The relevant presence of chemoheterotrophic bacteria in the de-sealed soil can be linked to its high mineralization degree, as evidenced by the observed low C/N ratio. These microorganisms, deriving energy from the oxidation of organic compounds, revealed the degradation of complex AVOCs as preferential metabolic pathway. Therefore, our data suggested the breakdown of various organic pollutants in a de-sealed and reused soil. The co-occurrence of multiple AVOCs in our soil samples made difficult to define a clear pattern of BVOC production. In the specified soil conditions, diverse interactions among AVOCs led to different degradation pathways, influenced by the types of microorganisms and enzymes present. Microbes may have broken down and transformed certain compounds, while using a specific AVOC as their primary substrate for energy and growth [106, 107]. Hence, the high concentration of AVOCs in soils sown with *Trifolium* might have been caused by a continuous organic C turnover, which further inhibited the VOC degradation [108, 109]. Interestingly, tetrahydrofuran, an AVOC previously detected in urban flowerbeds [61] and known as a potential groundwater contaminant, predominantly dominated the AVOC emissions from *Trifolium* soil samples. This compound is very susceptible to chemical and biological reactions and only a limited number of microbes capable of degrading tetrahydrofuran has been documented [110, 111]. Additionally, dimethyl disulfide was significantly higher in *Trifolium* samples as well. This sulfur-containing compound, known for its well-documented antifungal activity, has been isolated from soil inhabitants such as *Pseudomonas chloraphis* and *Stenotrophomonas rhizophila* [112, 113], which exhibited biocontrol activity against phytopathogenic fungi. Here it was positively correlated with *Ammoniphilus* genus (Firmicutes, Bacillales, Paenibacillaceae), a chemo-organotroph producing hydrogen sulfide taxon harboring ammonium-dependent bacteria [114, 116]. The dimethyl disulfide production was previously documented in *Bacillus* sp., as a plant growth promotion mechanism [117].

Conversely, our results showed lower concentrations of anthropogenic VOC classes in *C. cardunculus* associated soils, both with and without AMF, and in control samples compared to soils sown with *Trifolium*. This suggested the presence of a more efficient microbial consortium capable of aerobic degradation of these compounds, as reported by Yoshikawa et al. [107] and Wu et al. [118]. Indeed, Rhodobacterales and Gemmatimonadales associated to *Cynara*/AMF conditions were less abundant in *Trifolium* soils. Rhodobacterales is well known to include many members that have the ability to degrade a wide range of hydrocarbons [119], while Gemmatimonadales order was found to have a high efficiency of C utilization [120, 121]. A negative correlation between the arene class and *Ralstonia* genera indicated its potential role in VOC degradation as inferred by Ryan et al. [122]. Additionally, *Delftia*, *Ramlibacter*, and Chloroflexales were identified as key degraders of organic pollutants in AM fungi-colonized soils [65, 123–128] and here they were negatively correlated with VOCs. The VOC content in AM fungal inoculated soils showed a high absorption of VOCs by the soil microbial communities, likely due to improved pollutant breakdown, suggesting that the presence of AMF might enhance the soil capacity to degrade AVOCs, as proposed by Volante et al. [129]. This observed positive effect of inoculation may be attributed not only to the applied AM fungal species but also to their associated microbiota containing genes encoding catabolic enzymes [129, 130] or to the AMF-mediated changes observed in soil native microbial communities [97]. In addition, the phototroph-heterotroph partnership detected in inoculated soils may have significantly contributed to the overall degradation of AVOCs [131]. The association of AMF and *C. cardunculus* favored an initial rapid pollutant degradation by r-strategists, which created a favorable environment for K-strategists that then took over for sustained degradation once primary contaminants were reduced [132]. On the other hand, the low arene and furan concentrations from the soils of non-inoculated *C. cardunculus* might be explained by the presence of *Nocardioides*, a very versatile genus with an interesting potential in the degradation of various organic pollutants and bioremediation [133]. Additionally, the emissions of dichloromethane, quantitatively the most important chlorinated solvent industrially produced [134], were lower in all treatments compared to the control. Our metabarcoding results highlighted several genera known to degrade dichloromethane, including *Ralstonia*, *Vlycobacterium*, *Rhodococcus*, *Bacillus*, *Burkholderia*, *Micrococcus*, *Pseudomonas* [134]. In addition, a negative correlation was found in control soil samples between this AVOC and Vicinamibactereaceae family, part of the control soil core microbiome, and known for chemo-organoheterotroph metabolism and its ability to thrive in nutrient-poor, neutral, and heavy metal-tolerant environments [135]. The overall observed decrease in VOC concentration in our experimental conditions can serve as “proxy” for the efficiency of VOC degradation process, occurring in contaminated soils, as recently proposed [61].

Notably, in our study, the *Caulobacter* presence was observed exclusively in the core microbiome of control soils, revealing physiological adaptations to low nutrient conditions [136]. The ability of this bacterium, to outlive other bacterial organoheterotrophs in nutrient depleted, long-term incubations, has been already documented [137]. It was worth noting that this bacterium may be involved alone or in consortia (i.e., with *Pseudomonas*, *Burkholderia*, *Mycobacterium*) in

biodegradation of hydrocarbons or other organic pollutants [124, 138, 139] and these genera belong to highly abundant orders in our control conditions. The presence of *Caulobacter* only in the core microbiome of control soils underscored that a degraded soil may function as a reservoir of microbes involved in decontamination processes, which could be potentially exploited for the restoration of soil ecological functions [136].

Positive correlations have been found between several bacterial taxa and terpene/terpenoid classes especially in *C. cardunculus* inoculated soil. It is widely considered that terpenes are plant or fungal metabolites [140], but predicted terpene synthases were also found in bacteria belonging orders like Myxococcales, Burkholderiales, Rhizobiales, Sphingobacteriales, Pseudomonadales [141] detected in our experimental conditions. The identified microbial consortium may have utilized terpenes/terpenoids as C source and energy for the subsequent soil contaminants degradation [142, 144]. However, further studies on *bacterial terpenome* [145] and its role in bioremediation are required.

4.3 Synergistic effects within the mycorrhizosphere of AMF-inoculated plants contributed to soil nutrient availability

Interestingly, a high content of acetic acid was observed in the presence of AMF and several positive correlations between bacteria and this VOC class were identified. Various soil/rhizosphere-inhabiting microorganisms release different amounts of organic acids in the soil [146, 148]. The presence of some of them, such as *Bacillus* sp., *Lysinibacillus* sp., and *Pseudomonas* sp., strongly correlating with acetic acid, suggested that the production of this organic acid was occurring in the soil. Acetic acid production by microbes has been linked to an improved availability of phosphorus [149, 150]. In this line, *Bacillus*, *Lysinibacillus* and *Pseudomonas* taxa correlating with high level of acetic acid in soil of AMF-inoculated plants may be indicative of the presence of the so-called *mycorrhizosphere*, which is composed by different microbial communities supposed to have a synergistic effect with AMF on different processes, including phosphorus solubilization [151].

5. Conclusions

This study demonstrated the colonization of plant roots by AMF in a de-sealed and degraded soil inoculated with an AMF-based inoculum. No native AM fungal colonization was observed in non-inoculated pots. Inoculating a de-sealed and degraded soil with a commercial AM fungal inoculum and using cover plants like *Cynara cardunculus* restored soil health status, also by improving soil bacterial diversity. The presence of complex microbial consortia facilitated AVOC degradation, highlighting the bioremediation potential of this approach in urban soils. Chemoheterotrophic microorganisms were key players in pollutant breakdown. The findings supported the “Microbiome *Rewilding* Hypothesis” showing that reintroducing beneficial microorganisms and plants can create a dynamic, enriched soil ecosystem that fosters microbial diversity and resilience against urban degradation.

Acknowledgements We thank the Municipality of Prato for coordinating the activities and providing access to the sites. AC is funded by a grant within WATDEV project (FOOD/2021/425-767). MAC fellowship was funded by the PRIMA RESCHEDULE project (Italian MUR DD 1 293/2021).

Author contributions Fabiano Sillo, Alessandro Zaldei, Beniamino Gioli, Rita Baraldi, Raffaella Balestrini made substantial contributions to the conception of the work; Maria Alexandra Cucu, Luisa Neri, Fabiano Sillo, Elisa Zampieri, Alice Calvo, Luca Giovannini, Cinzia De Benedictis, Alessandro Zaldei, Beniamino Gioli, Rita Baraldi, Raffaella Balestrini made substantial contributions to the acquisition, analysis, or interpretation of data; Maria Alexandra Cucu, Fabiano Sillo, Elisa Zampieri drafted the manuscript; all the authors, approved the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding This work was supported by the Urban Innovative Actions (UIA) program of the European Commission, under the project PUJ—Prato Urban Jungle, project number UIA04-176.

Data availability Sequence data that support the findings of this study have been deposited with the primary accession code PRJNA1 133247.

Code availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Egerer M, Haase D, McPhearson T, Frantzeskaki N, Andersson E, Nagendra H, Ossola A. Urban change as an untapped opportunity for climate adaptation. *NPJ Urban Sustain.* 2021;1:22. <https://doi.org/10.1038/s42949-021-00024-y>.
- Urban MC, Alberti M, De Meester L, Zhou Y, Verrelli BC, Szulkin M, Schmidt C, Savage AM, Roberts P, Rivkin LR, Palkovacs EP, Munshi-South J, Malesis AN, Harris NC, Gotanda KM, Garroway CJ, Diamond SE, Des Roches S, Charmanier A, Brans KI. Interactions between climate change and urbanization will shape the future of biodiversity. *Nat Clim Chang.* 2024;14:436–47. <https://doi.org/10.1038/s41558-024-01996-2>.
- Pugnaire FI, Morillo JA, Peñuelas J, Reich PB, Bardgett RD, Gaxiola A, Wardle DA, van der Putten WH. Climate change effects on plant-soil feedbacks and consequences for biodiversity and functioning of terrestrial ecosystems. *Sci Adv.* 2019;5:1834. <https://doi.org/10.1126/sciadv.aaz1834>.
- O'Riordan R, Davies J, Stevens C, Quinton JN, Boyko C. The ecosystem services of urban soils: a review. *Geoderma.* 2021;385: 114867. <https://doi.org/10.1016/j.geoderma.2020.114867>.
- Shahane AA, Shivay YS. Soil health and its improvement through novel agronomic and innovative approaches. *Front Agron.* 2021;3: 680456. <https://doi.org/10.3389/fagro.2021>.
- United Nations, Department of Economic and Social Affairs, Population Division (UNEP). World urbanization prospects: the 2014 revision, highlights; 2014. (ST/ESA/SER.A/352)
- Food and Agriculture Organization (FAO) and Intergovernmental Technical Panel on Soils (ITPS). The status of the world's soil resources (main report). Rome: Food and Agriculture Organization of the United Nations; 2015.
- Marcotullio PJ, Braimoh AK, Onishi T. The impact of urbanization on soils. In: Braimoh AK, Vlek PLG, editors. Land use and soil resources. Dordrecht: Springer; 2008. https://doi.org/10.1007/978-1-4020-6778-5_10.
- United Nations, Department of Economic and Social Affairs, Population Division (UNEP). Global environmental outlook—GEO-6: healthy planet, healthy people. Cambridge University Press; 2021. <https://doi.org/10.1017/9781108627146>.
- Peroni F, Pappalardo SE, Facchinelli F, Crescini E, Munafò M, Hodgson ME, De Marchi M. How to map soil sealing, land take and impervious surfaces? A systematic review. *Environ Res Lett.* 2022. <https://doi.org/10.1088/1748-9326/ac26eb>.
- Tobias S, Conen F, Duss A, Wenzel L, Buser C, Alewell C. Soil sealing and unsealing: state of the art and examples. *Land Degrad Dev.* 2018;2018(29):2015–24. <https://doi.org/10.1002/ldr.2919>.
- Scalenghe R, Ajmone MF. The anthropogenic sealing of soil in urban areas. *Landsc Urban Plan.* 2009;90:1–10. <https://doi.org/10.1016/j.landurbplan.2008.10.011>.
- Piotrowska-Długosz A, Charzyński P. The impact of the soil sealing degree on microbial biomass, enzymatic activity, and physicochemical properties in the Ekranic Technosols of Toruń (Poland). *J Soil Sediment.* 2015;15:47–59. <https://doi.org/10.1007/s11368-014-0963-8>.
- Tóth G, Ivits E, Prokop G, Gregor M, Fons-Esteve J, Milego Agràs R, Mancosu E. Impact of soil sealing on soil carbon sequestration, water storage potentials, and biomass productivity in functional urban areas of the European Union and the United Kingdom. *Land.* 2022;11:840. <https://doi.org/10.3390/land11060840>.
- Maienza A, Ungaro F, Baronti S, Colzi I, Giagnoni L, Gonnelli C, Renella G, Ugolini F, Calzolari C. Biological restoration of urban soils after de-sealing interventions. *Agriculture.* 2021;11:190. <https://doi.org/10.3390/agriculture11030190>.
- Caselli B, Ceci M, De Noia I, Garda E, Zazzi M. Towards the integration of soil desealing in the urban areas' transformation processes. In: Marucci A, Zullo F, Fiorini L, Saganeiti L, editors. Innovation in urban and regional planning lecture notes in civil engineering, vol. 467. Cham: Springer; 2024. https://doi.org/10.1007/978-3-031-54118-6_27.
- Barbato RA, Reynolds CM. Bioremediation of contaminated soils. In: Gentry TJ, Fuhrmann JJ, Zuberer DA, editors. Principles and applications of soil microbiology. 3rd ed. Londn: Elsevier; 2022. p. 607–31. <https://doi.org/10.1016/B978-0-12-820202-9.00022-8>.
- Chu C, Zhu L. Paving the way toward soil safety and health: current status, challenges, and potential solutions. *Front Environ Sci Eng.* 2024;18:74. <https://doi.org/10.1007/s11783-024-1834-1>.
- Kabisch N, Strohbach M, Haase D, Kronenberg J. Urban green space availability in European cities. *Ecol Indic.* 2016;70:586–96. <https://doi.org/10.1016/j.ecolind.2016.02.029>.
- Chicca I, Becarelli S, Di Gregorio S. Microbial involvement in the bioremediation of total petroleum hydrocarbon polluted soils: challenges and perspectives. *Environ.* 2022;9:52. <https://doi.org/10.3390/environments9040052>.
- Asmelash F, Bekele T, Birhane E. The potential role of arbuscular mycorrhizal fungi in the restoration of degraded lands. *Front Microbiol.* 2016;7:1095. <https://doi.org/10.3389/fmicb.2016.01095>.
- Zhang J, Zhao R, Li X, Zhang J. Potential of arbuscular mycorrhizal fungi for soil health: a review. *Pedosphere.* 2024;34:279–88. <https://doi.org/10.1016/j.pedosph.2024.02.002>.
- Lehmann A, Barto EK, Powell JR, Rillig MC. Mycorrhizal responsiveness trends in annual crop plants and their wild relatives—a meta-analysis on studies from 1981 to 2010. *Plant Soil.* 2012;355:231–50. <https://doi.org/10.1007/s11104-011-1095-1>.
- Balestrini R, Brunetti C, Chitarra W, Nerva L. Photosynthetic traits and nitrogen uptake in crops: which is the role of arbuscular mycorrhizal fungi? *Plant.* 2020;9:1105. <https://doi.org/10.3390/plants9091105>.
- Cabello MN. Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). *FEMS Microbiol Ecolol.* 1997;22:233–6. <https://doi.org/10.1111/j.1574-6941.1997.tb00375.x>.

26. Sieverding E. (1991). Vesicular-arbuscular mycorrhiza management in tropical agrosystems, 371 pp. Deutsche Gesellschaft für Technische Zusammenarbeit, GTZ No. 224. Eschborn
27. Gaur A, Adholeya A. Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. *Curr Sci.* 2004;86:528–34.
28. Javadi A. Importance of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. In: Khan M, Zaidi A, Goel R, Musarrat J, editors. *Bio management of metal-contaminated soils. Environmental pollution*, vol. 20. Dordrecht: Springer; 2011. https://doi.org/10.1007/978-94-007-1914-9_5.
29. Patel S, Sharma A, Batra NG. Arbuscular Mycorrhizal fungi-Assisted Bioremediation of heavy metals: a reevaluation. In: Arora S, Kumar A, Ogita S, Yau YY, editors. *Innovations in environmental biotechnology*. Singapore: Springer; 2022. https://doi.org/10.1007/978-981-16-4445-0_32.
30. Okrasinska A, Decewicz P, Majchrowska M, Dziewit Ł, Muszewska A, Dolatabadi S, Kruszewski Ł, Błocka Z, Pawłowska J. Marginal lands and fungi—linking the type of soil contamination with fungal community composition. *Environ Microbiol.* 2022;24:3809–25. <https://doi.org/10.1111/1462-2920.16007>.
31. Crişan I, Balestrini R, Pagliarani C. The current view on heavy metal remediation: the relevance of the plant interaction with arbuscular mycorrhizal fungi. *Plant Stress.* 2024;12: 100439. <https://doi.org/10.1016/j.stress.2024.100439>.
32. Arriagada CA, Herrera MA, García-Romera I, Ocampo JA. Tolerance to Cd of soybean (*Glycine max*) and eucalyptus (*Eucalyptus globulus*) inoculated with arbuscular mycorrhizal and saprobe fungi. *Symbiosis.* 2004;36:285–99.
33. Arriagada CA, Herrera MA, Borie F, Ocampo JA. Contribution of arbuscular mycorrhizal and saprobe fungi to the aluminum resistance of *Eucalyptus globulus*. *Water Air Soil Pollut.* 2007;2007(182):383–94. <https://doi.org/10.1007/s11270-007-9349-5>.
34. Alarcón A, Davies F Jr, Autenrieth R, Zuberer DA. Arbuscular mycorrhiza and petroleum-degrading microorganisms enhance phytoremediation of petroleum-contaminated soil. *Int J Phytoremediat.* 2008;10:251–63. <https://doi.org/10.1080/15226510802096002>.
35. Yu XZ, Wu SC, Wu FY, Wong MH. Enhanced dissipation of PAHs from soil using mycorrhizal ryegrass and PAH-degrading bacteria. *J Hazard Mat.* 2011;186:1206–17. <https://doi.org/10.1016/j.jhazmat.2010.11.116>.
36. Xiao X, Chen H, Si C, Wu L. Influence of biosurfactant-producing strain *Bacillus subtilis* BS1 on the mycoremediation of soils contaminated with phenanthrene. *Int Biodeterior Biodegrad.* 2012;75:36–42. <https://doi.org/10.1016/j.ibiod.2012.09.002>.
37. Llugany M, Miralles R, Corrales I, Barceló JPC. *Cynara cardunculus* a potentially useful plant for remediation of soils polluted with cadmium or arsenic. *J Geochem Explor.* 2012;123:122–7. <https://doi.org/10.1016/j.gexplo.2012.06.016>.
38. Capozzi F, Sorrentino MC, Caporale AG, Fiorentino N, Giordano S, Spagnuolo V. Exploring the phytoremediation potential of *Cynara cardunculus*: a trial on an industrial soil highly contaminated by heavy metals. *Environ Sci Poll Res.* 2020;27:9075–84. <https://doi.org/10.1007/s11356-019-07575-9>.
39. Pappalardo HD, Toscano V, Puglia GD, Genovese C, Raccuia SA. *Cynara cardunculus* L. as a multipurpose crop for plant secondary metabolites production in marginal stressed lands. *Front Plant Sci.* 2020;11:240. <https://doi.org/10.3389/fpls.2020.00240>.
40. Liu C, Lin H, Dong Y, Li B, Liu Y. Investigation on microbial community in remediation of lead-contaminated soil by *Trifolium repens* L. *Ecotoxicol Environ Saf.* 2018;165:52–60. <https://doi.org/10.1016/j.ecoenv.2018.08.054>.
41. Turo KJ, Gardiner MM. From potential to practical: conserving bees in urban public green spaces. *Front Ecol Environ.* 2019;17:167–75. <https://doi.org/10.1002/fee.2015>.
42. Pilon-Smits E. Phytoremediation. *Ann Rev Plant Biol.* 2005;56:15–39. <https://doi.org/10.1146/annurev.arplant.56.032604.144214>.
43. Tangahu BV, Sheikh Abdullah SR, Basri H, Idris M, Anuar N, Mukhlis M. A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. *Int J Chem Eng.* 2011;2011:31.
44. Nannipieri P, Ascher J, Ceccherini MT, Landi L, Pietramellara G, Renella G. Microbial diversity and soil functions. *Europ J Soil Sci.* 2003;54:655–70. <https://doi.org/10.1046/j.1351-0754.2003.0556.x>.
45. Fierer N, Jackson RB. The diversity and biogeography of soil bacterial communities. *PNAS.* 2006;103:626–31. <https://doi.org/10.1073/pnas.0507535103>.
46. Prosser JI. Exploring soil microbial communities: opportunities for soil ecology research. *Soil Ecol Lett.* 2019;1:1–2. <https://doi.org/10.1007/s42832-019-0001-2>.
47. Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH. Going back to the roots: the microbial ecology of the rhizosphere. *Nat Rev Microbiol.* 2013;11:789–99. <https://doi.org/10.1038/nrmicro3109>.
48. Philippot L, Chenu C, Kappler A, Rillig MC, Fierer N. The interplay between microbial communities and soil properties. *Nat Rev Microbiol.* 2024;22:226–39. <https://doi.org/10.1038/s41579-023-00980-5>.
49. Malhautier L, Khammar N, Bayle S, Fanlo J-L. Biofiltration of volatile organic compounds. *App Microbiol Biotech.* 2005;68:16–22. <https://doi.org/10.1007/s00253-005-1960-z>.
50. Bastida F, Hernández T, García C. Soil degradation and rehabilitation: Microorganisms and functionality. In: Buscot F, Varma A, editors. *Soil Biology*, vol. 19. Springer; 2010. p. 285–306. https://doi.org/10.1007/978-3-642-04043-6_13.
51. Lee SH, Jin HM, Lee HJ, Kim JM, Jeon CO. Complete genome sequence of the BTEX-degrading bacterium *Pseudoxanthomonas spadix* BD-a59. *J Bacteriol.* 2012;194:544. <https://doi.org/10.1128/JB.06436-11>.
52. Kertesz MA, Kawasaki A, Stolz A. Aerobic hydrocarbon-degrading alphaproteobacteria: sphingomonadales. In: McGenity T, editor. *Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes. Handbook of hydrocarbon and lipid microbiology*. Cham: Springer; 2019. https://doi.org/10.1007/978-3-030-14796-9_9.
53. Lee Y, Lee Y, Jeon CO. Biodegradation of naphthalene, BTEX, and aliphatic hydrocarbons by *Paraburkholderia aromaticivorans* BN5 isolated from petroleum-contaminated soil. *Sci Rep.* 2019;9:860. <https://doi.org/10.1038/s41598-018-36165-x>.
54. Mbachu AE, Chukwura EI, Mbachu NA. Role of microorganisms in the degradation of organic pollutants: a review. *Energy Environ Eng.* 2020;7:1–11. <https://doi.org/10.13189/eee.2020.070101>.
55. Eze MO. Metagenome analysis of a hydrocarbon-degrading bacterial consortium reveals the specific roles of BTEX biodegraders. *Genes.* 2021. <https://doi.org/10.3390/genes12010098>.
56. Lehmann J, Bossio DA, Kögel-Knabner I, Rillig MC. The concept and future prospects of soil health. *Nat Rev Earth Env.* 2020;1:544–53. <https://doi.org/10.1038/s43017-020-0080-8>.

58. Raghavendra M, Sharma MP, Ramesh A, Richa A, Billore SD, Verma RK. Soil health indicators: methods and applications. In: Rakshit A, Ghosh S, Chakraborty S, Philip V, Datta A, editors. *Soil analysis: recent trends and applications*. Springer; 2020. p. 13–26. https://doi.org/10.1007/978-981-15-2039-6_13.
59. Chang T, Feng G, Paul V, Adeli A, Brooks JP. Soil health assessment methods: Progress, applications and comparison. In: Sparks DL, editor. *Advances in agronomy*, vol. 172. Academic Press; 2022. p. 129–210. <https://doi.org/10.1016/bs.agron.2021.10.002>.
60. Juhos K, Nugroho PA, Jakab G, Prettl N, Kotroczo Z, Szigeti N, Szalai Z, Madarász B. A comprehensive analysis of soil health indicators in a long-term conservation tillage experiment. *Soil Use Manage*. 2024;40: e12942. <https://doi.org/10.1111/sum.12942>.
61. Neri L, Sillo F, Zampieri E, Lumini E, Marturano G, Esposti CD, Petruzzelli G, Gioli B, Zaldei A, Baraldi R, Balestrini R. A combined analysis based on microbial communities and volatile organic compounds as a tool to study soil quality in an urban environment. *Pedosphere*. 2023;33:670–5. <https://doi.org/10.1016/j.pedsph.2023.01.013>.
62. Sillo F, Neri L, Calvo A, Zampieri E, Petruzzelli G, Ferraris I, Delledonne M, Zaldei A, Gioli B, Baraldi R, Balestrini R. Correlation between microbial communities and volatile organic compounds in an urban soil provides clues on soil quality towards sustainability of city flowerbeds. *Heliyon*. 2024. <https://doi.org/10.1016/j.heliyon.2023.e23594>.
63. Leff JW, Fierer N. Volatile organic compound (VOC) emissions from soil and litter samples. *Soil Biol Biochem*. 2008;40:1629–36. <https://doi.org/10.1016/j.soilbio.2008.01.018>.
64. Peñuelas J, Asensio D, Tholl D, Wenke K, Rosenkranz M, Piechulla B, Schnitz JP. Biogenic volatile emissions from the soil. *Plant Cell Environ*. 2014;37:1866–91.
65. Li AJ, Pal VK, Kannan K. A review of environmental occurrence, toxicity, biotransformation and biomonitoring of volatile organic compounds. *Environ Chem Ecotox*. 2021;3:91–116. <https://doi.org/10.1016/j.enceco.2021.01.001>.
66. Varjani SJ, Gnansounou E, Pandey A. Comprehensive review on toxicity of persistent organic pollutants from petroleum refinery waste and their degradation by microorganisms. *Chemosphere*. 2017;188:280–91. <https://doi.org/10.1016/j.chemosphere.2017.09.005>.
67. Mangotra A, Singh SK. Volatile organic compounds: a threat to the environment and health hazards to living organisms—a review. *J Biotech*. 2024;382:51–69. <https://doi.org/10.1016/j.jbiotec.2023.12.013>.
68. Insam H, Seewald MSA. Volatile organic compounds (VOCs) in soils. *Biol Fert Soil*. 2010;46:199–213. <https://doi.org/10.1007/s00374-010-0442-3>.
69. Mishra S, Lin Z, Pang S, Zhang W, Bhatt P, Chen S. Recent advanced technologies for the characterization of xenobiotic-degrading microorganisms and microbial communities. *Front Bioeng Biotech*. 2021;9: 632059. <https://doi.org/10.3389/fbioe.2021.632059>.
70. Giovannetti M, Mosse B. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol*. 1980;84:489–500. <https://doi.org/10.1111/j.1469-8137.1980.tb04556.x>.
71. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J*. 2017;11:2639–43. <https://doi.org/10.1038/ismej.2017.119>.
72. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. *Nat Biotech*. 2019;37:852–7. <https://doi.org/10.1038/s41587-019-0209-9>.
73. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2012;41:D590–6. <https://doi.org/10.1093/nar/gks1219>.
74. Abarenkov K, Nilsson RH, Larsson K-H, Taylor AFS, May TW, Frøslev TG, Pawłowska J, Lindahl B, Pöldmaa K, Truong C, Vu D, Hosoya T, Niskanen T, Piirmann T, Ivanov F, Zirk A, Peterson M, Cheeke TE, Ishigami Y, Jansson AT, Jeppesen TS, Kristiansson E, Mikryukov V, Miller JT, Oono R, Ossandon FJ, Paupério J, Saar I, Schigel D, Suija A, Tedersoo L, Kõljalg U. The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: Sequences, taxa and classifications reconsidered. *Nucleic Acids Res*. 2024;52:D791–7. <https://doi.org/10.1093/nar/gkad1039>.
75. Opik M, Vanatoa A, Vanatoa E, Moora M, Davison J, Kalwij JM, Reier U, Zobel M. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytol*. 2010;188:223–41. <https://doi.org/10.1111/j.1469-8137.2010.03334.x>.
76. Chong J, Liu P, Zhou G, Xia J. Using Microbiome Analyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat Prot*. 2020;15:799–821. <https://doi.org/10.1038/s41596-019-0264-1>.
77. Baraldi R, Chieco C, Neri L, Facini O, Rapparini F, Morrone L, Rotondi A, Carriero G. An integrated study on air mitigation potential of urban vegetation: from a multi-trait approach to modeling. *Urban For Urban Green*. 2019;41:127–38. <https://doi.org/10.1016/j.ufug.2019.03.020>.
78. Louca S, Parfrey LW, Doebeli M. Decoupling function and taxonomy in the global ocean microbiome. *Sci*. 2016;353:1272–7. <https://doi.org/10.1126/science.aaf4507>.
79. Smit E, Leeftang P, Gommans S, van den Broek J, van Mil S, Wernars K. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl Environ Microbiol*. 2001;67:2284–91. <https://doi.org/10.1128/AEM.67.5.2284-2291.2001>.
80. Trivedi P, Delgado-Baquerizo M, Anderson IC, Singh BK. Response of soil properties and microbial communities to agriculture: implications for primary productivity and soil health indicators. *Front Plant Sci*. 2016;7:990. <https://doi.org/10.3389/fpls.2016.00990>.
81. De Vries FT, Hoffland E, van Eekeren N, Brussaard L, Bloem J. Fungal/bacterial ratios in grasslands with contrasting nitrogen management. *Soil Biol Biochem*. 2006;38:2092–103. <https://doi.org/10.1016/j.soilbio.2006.01.008>.
82. Djemiel C, Dequiedt S, Bailly A, Tripied J, Lelièvre M, Horrigue W, Jolivet C, Bispo A, Saby N, Valé M, Maron PA, Ranjard L, Terrat S. Biogeographical patterns of the soil fungal: bacterial ratio across France. *Mosphere*. 2023;8:e00365–e423. <https://doi.org/10.1128/msphe.re.00365-23>.
83. Lemfack MC, Gohlke BO, Toguem S, Preissner S, Piechulla B, Preissner R. mVOC 2.0: a database of microbial volatiles. *Nucleic Acids Res*. 2017;46:1261–5. <https://doi.org/10.1093/nar/gkx1016>.
84. Schulz S, Dickschat JS. Bacterial volatiles: the smell of small organisms. *Chem Inform*. 2007. <https://doi.org/10.1002/chin.200745263>.
85. Giorgio A, De Stradis A, Lo Cantore P, Iacobellis NS. Biocide effects of volatile organic compounds produced by potential biocontrol rhizobacteria on *Sclerotinia sclerotiorum*. *Front Microbiol*. 2015;6:1056. <https://doi.org/10.3389/fmicb.2015.01056>.
86. Vaishnav A, Kumari S, Jain S, Varma A, Choudhary DK. Putative bacterial volatile-mediated growth in soybean (*Glycine max* L. Merrill) and expression of induced proteins under salt stress. *J Appl Microbiol*. 2015;119:539–51. <https://doi.org/10.1111/jam.12866>.

- 87.
88. Goddard MA, Dougill AJ, Benton TG. Scaling up from gardens: biodiversity conservation in urban environments. *Trends Ecol Evol.* 2010;25:9 <https://doi.org/10.1016/j.tree.2009.07.016>.
89. Seto KC, Güneralp B, Hutyra LR. Global forecasts of urban expansion to 2030 and direct impacts on biodiversity and carbon pools. *PNAS.* 2012;109:16083–8. <https://doi.org/10.1073/pnas.1211658109>.
90. Mills JG, Weinstein P, Gellie NJC, Weyrich LS, Lowe AJ, Breed M. Urban habitat restoration provides a human health benefit through microbiome rewilding: the Microbiome Rewilding Hypothesis. *Restor Ecol.* 2017;25:866–72. <https://doi.org/10.1111/rec.12610>.
91. Renella G. Evolution of physico-chemical properties, microbial biomass and microbial activity of an urban soil after de-sealing. *Agriculture.* 2020;10:596. <https://doi.org/10.3390/agriculture10120596>.
92. Turnau K, Miszals Z, Trouvelot A, Bonfante P, Gianinazzi S. *Oxalis acetosella* as monitoring plant on highly polluted soils. In: Azcón-Aguilar C, Barea JM, editors. Mycorrhizas in integrated systems: from genes to plant development. European Commission: Luxembourg; 1996. p. 483–6.
93. del Val C, Barea JM, Azcón-Aguilar C. Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. *Appl Environ Microbiol.* 1999;65:718–23. <https://doi.org/10.1128/AEM.65.2.718-723.1999>.
94. Jacquot-Plumey E, Van Tuinen D, Chatagnier O, Gianinazzi S, Gianinazzi-Pearson V. 25S rDNA-based molecular monitoring of glomalean fungi in sewage sludge-treated field plots. *Environ Microbiol.* 2001;3:525–31. <https://doi.org/10.1046/j.1462-2920.2001.00219.x>.
95. Schnoor TK, Lekberg Y, Rosendahl S, Olsson PA. Mechanical soil disturbance as a determinant of arbuscular mycorrhizal fungal communities in semi-natural grassland. *Mycorrhiza.* 2011;21:211–20. <https://doi.org/10.1007/s00572-010-0325-3>.
96. Lauber CL, Hamady M, Knight R, Fierer N. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microbiol.* 2009;75:5111–20. <https://doi.org/10.1128/AEM.00335-09>.
97. Rousk J, Brookes PC, Baath E. Contrasting soil pH effects on fungal and bacterial growth suggest functional redundancy in carbon mineralization. *Appl Environ Microbiol.* 2009;2009(75):1589–96. <https://doi.org/10.1128/AEM.02775-08>.
98. Nuñez NF, Maggia L, Stenger PL, Lelievre M, Letellier K, Gigante S, Manez A, Mournet P, Ripoll J, Carriconde F. Potential of high-throughput eDNA sequencing of soil fungi and bacteria for monitoring ecological restoration in ultramafic substrates: the case study of the New Caledonian biodiversity hotspot. *Ecol Eng.* 2021;173: 106416. <https://doi.org/10.1016/j.ecoleng.2021.106416>.
99. Solís-Ramos LY, Coto-López C, Andrade-Torres A. Role of arbuscular mycorrhizal symbiosis in remediation of anthropogenic soil pollution. *Symbiosis.* 2021;84:321–36. <https://doi.org/10.1007/s13199-021-00774-4>.
100. Jiao Y, Kramshøj M, Davie-Martin CL, Albers CN, Rinnan R. Soil uptake of VOCs exceeds production when VOCs are readily available. *Soil Biol Biochem.* 2023;185: 109153. <https://doi.org/10.1016/j.soilbio.2023.109153>.
101. Bacosá HP, Mabuhay-Omar JA, Balisco RT, Omar DM Jr, Inoue C. Biodegradation of binary mixtures of octane with benzene, toluene, ethylbenzene or xylene (BTEX): Insights on the potential of *Burkholderia*, *Pseudomonas*, and *Cupriavidus* isolates. *World J Microbiol Biotechnol.* 2021;37:122. <https://doi.org/10.1007/s11274-021-03093-4>.
102. Hocinat A, Boudemagh A, Ali-Khodja H, Medjemadj M. Aerobic degradation of BTEX compounds by *Streptomyces* species isolated from activated sludge and agricultural soils. *Arch Microbiol.* 2020;202:2481–92. <https://doi.org/10.1007/s00203-020-01970-4>.
103. Zhou Y, Huang H, Shen DD. Multi-substrate biodegradation interaction of 1,4-dioxane and BTEX mixtures by *Acinetobacter baumannii* DD1. *Biodegradation.* 2016;27:37–46. <https://doi.org/10.1007/s10532-015-9753-2>.
104. Wongbunmak A, Khiawjan S, Supphantharika M, Pongtharangkul T. BTEX- and naphthalene-degrading bacterium *Microbacterium esteraromaticum* strain SBS1-7 isolated from estuarine sediment. *J Hazard Mater.* 2017;339:82–90. <https://doi.org/10.1016/j.jhazmat.2017.03.041>.
105. Wongbunmak A, Khiawjan S, Supphantharika M, Pongtharangkul T. BTEX biodegradation by *Bacillus amyloliquefaciens* subsp. plantarum W1 and its proposed BTEX biodegradation pathways. *Sci Rep.* 2020;10:17408. <https://doi.org/10.1038/s41598-020-74570-3>.
106. Son J, Lee H, Kim M, Kim DU, Ka JO. *Massilia aromanticivorans* sp. nov., a BTEX degrading bacterium isolated from Arctic soil. *Curr Microbiol.* 2021;78:2143–50. <https://doi.org/10.1007/s00284-021-02379-y>.
107. Orro A, Cappelletti M, Dursi P, Milanese L, Canito AD, Zampolli J, Collina E, Decorosi F, Viti C, Fedi S, Presentato A, Zannoni D, Fedi S. Genome and phenotype microarray analyses of *Rhodococcus* sp. BCP1 and *Rhodococcus opacus* R7: genetic determinants and metabolic abilities with environmental relevance. *PLoS ONE.* 2015. <https://doi.org/10.1371/journal.pone.0139467>.
108. Imron MF, Kurniawan SB, Ismail NI, Abdullah SRS. Future challenges in diesel biodegradation by bacteria isolates: a review. *J Clean Prod.* 2020;251: 119716. <https://doi.org/10.1016/j.jclepro.2019.119716>.
109. Yoshikawa M, Zhang M, Toyota K. Biodegradation of volatile organic compounds and their effects on biodegradability under co-existing conditions. *Microbes Environ.* 2017. <https://doi.org/10.1264/jsme2.ME16188>.
110. Rinnan R, Albers CN. Soil uptake of volatile organic compounds: ubiquitous and underestimated? *J Geophys Res.* 2020;125:e2020JG005773.
111. Xu X, Liu W, Tian S, Wang W, Qi Q, Jiang P, Gao X, Li F, Li H, Yu H. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: a perspective analysis. *Front Microbiol.* 2018;9:2885.
112. Bernhardt D, Diekmann H. Degradation of dioxane, tetrahydrofuran and other cyclic ethers by an environmental *Rhodococcus* strain. *Appl Microbiol Biotech.* 1991;36:120–3. <https://doi.org/10.1007/BF00164711>.
113. Qi M, Huang H, Zhang Y, Wang H, Li H, Lu Z. Novel tetrahydrofuran (THF) degradation-associated genes and cooperation patterns of a THF-degrading microbial community as revealed by metagenomic. *Chemosphere.* 2019;231:173–83. <https://doi.org/10.1016/j.chemosphere.2019.05.137>.
114. Ren H, Li H, Wang H, Huang H, Lu Z. Biodegradation of Tetrahydrofuran by the newly isolated filamentous fungus *Pseudallescheria boydii* ZM01. *Microorganisms.* 2020;8:1190. <https://doi.org/10.3390/microorganisms8081190>.
115. Raio A, Puopolo G. *Pseudomonas chlororaphis* metabolites as biocontrol promoters of plant health and improved crop yield. *World J Microbiol Biotechnol.* 2021;37:99. <https://doi.org/10.1007/s11274-021-03063-w>.
116. Zaitsev GM, Tsitko IV, Rainey FA, Trotsenko YA, Uotila JS, Stackebrandt E, Salkinoja-Salonen MS. New aerobic ammonium-dependent obligately oxalotrophic bacteria: description of *Ammoniphilus oxalaticus* gen. nov., sp. nov. and *Ammoniphilus oxalivorans* gen. nov., sp. nov. *Int J Syst Evol Microbiol.* 1998;48:151–63. <https://doi.org/10.1099/00207713-48-1-151>.
117. Lin SY, Wu YH, Hameed A, Liu YC, Young CC. *Ammoniphilus resiniae* sp. nov., an endospore-forming bacterium isolated from resin fragments. *Int J Syst Evol Microbiol.* 2016;66:3010–6. <https://doi.org/10.1099/ijsem.0.001137>.

118. Meldau DG, Meldau S, Hoang LH, Underberg S, Wünsche H, Baldwin IT. Dimethyl disulfide produced by the naturally associated bacterium *Bacillus* sp. B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition. *Plant Cell*. 2013;25:2731–47. <https://doi.org/10.1105/tpc.113.114744>.
119. Wu H-J, Du X-Y, Xiao W-J, Zheng J, Song J-Y, Xie J-C. Metagenomic analysis reveals specific BTEX degrading microorganisms of a bacterial consortium. *AMB Expr*. 2023. <https://doi.org/10.1186/s13568-023-01541-y>.
120. Yavari-Bafghi M, Rezaei Somee M, Amoozegar MA, Dastgheib SM, Shavandi M. Genome-resolved analyses of oligotrophic groundwater microbial communities along phenol pollution in a continuous-flow biodegradation model system. *Front Microbiol*. 2023. <https://doi.org/10.3389/fmicb.2023.1147162>.
121. Koblížek M, Dachev M, Bina D, Nupur Piwosz K, Kaftan D. Utilization of light energy in phototrophic Gemmatimonadetes. *J Photochem Photobiol B: Biol*. 2020;213: 112085. <https://doi.org/10.1016/j.jphotobiol.2020.112085>.
122. Mujakić I, Piwosz K, Koblížek M. Phylum Gemmatimonadota and its role in the environment. *Microorganisms*. 2022;10:151. <https://doi.org/10.3390/microorganisms10010151>.
123. Ryan MP, Pembroke JT, Adley CC. *Ralstonia pickettii* in environmental biotechnology: potential and applications. *J Appl Microbiol*. 2007;103:754–64. <https://doi.org/10.1111/j.1365-2672.2007.03361.x>.
124. Fouad FA, Youssef DG, Shahat FM, Abd El-Ghany MN. Role of microorganisms in biodegradation of pollutants. In: Ali GAM, Makhoulf ASH, editors. *Handbook of biodegradable materials*. Cham: Springer; 2023. https://doi.org/10.1007/978-3-031-09710-2_11.
125. Vásquez-Piñeros MA, Martínez-Lavanchy PM, Jehmlich N, Pieper DH, Rincón CA, Harms H, Junca H, Heipieper HJ. *Delftia* sp. LCW, a strain isolated from a constructed wetland shows novel properties for dimethylphenol isomers degradation. *BMC Microbiol*. 2018. <https://doi.org/10.1186/s12866-018-1255-z>.
126. Krzmarzick MJ, Cray BB, Harding JJ, Oyerinde OO, Leri AC, Myneni SCB, Novak PJ. Natural niche for organohalide-respiring *Chloroflexi*. *Appl Environ Microbiol*. 2012;78:7856–62. <https://doi.org/10.1128/AEM.01723-12>.
127. Ghosal D, Ghosh S, Dutta TK, Ahn Y. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. *Front Microbiol*. 2016;7:1369. <https://doi.org/10.3389/fmicb.2016.01369>.
128. Freches A, Fradinho JC. The biotechnological potential of the *Chloroflexota* phylum. *Appl Environ Microbiol*. 2023;89:e01756-e1823. <https://doi.org/10.1128/aem.01756-23>.
129. Silva DEO, Costa RM, Campos JR, Rocha SMB, de Araujo Pereira AP, Melo VMM, Oliveira FAS, de Alcantara NF, Mende LW, Araujo ASF. Short-term restoration practices change the bacterial community in degraded soil from the Brazilian semiarid. *Sci Rep*. 2024;14:6845. <https://doi.org/10.1038/s41598-024-57690-y>.
130. Volante A, Lingua G, Cesaro P, Cresta A, Puppo M, Ariati L, Berta G. Influence of three species of arbuscular mycorrhizal fungi on the persistence of aromatic hydrocarbons in contaminated substrates. *Mycorrhiza*. 2005;16:43–50. <https://doi.org/10.1007/s00572-005-0012-y>.
131. Agnolucci M, Battini F, Cristani C, Giovannetti M. Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. *Biol Fertil Soils*. 2015;51:379–89. <https://doi.org/10.1007/s00374-014-0989-5>.
132. Abed RMM. Phototroph-heterotroph oil-degrading partnerships. In: McGenity T, editor. *Microbial communities utilizing hydrocarbons and lipids: members, metagenomics and ecophysiology Handbook of hydrocarbon and lipid microbiology*. Cham: Springer; 2019. https://doi.org/10.1007/978-3-319-60063-5_15-1.
133. Brzeszcz J, Steliga T, Kapusta P, Turkiewicz A, Kaszycki P. r-strategist versus K-strategist for the application in bioremediation of hydrocarbon-contaminated soils. *Int Biodeterior Biodegrad*. 2016;106:41–52. <https://doi.org/10.1016/j.ibiod.2015.10.001>.
134. Ma Y, Wang J, Liu Y, Wang X, Zhang B, Zhang W, Chen T, Liu G, Xue L, Cui X. Nocardioideae: “Specialists” for hard-to-degrade pollutants in the environment. *Molecules*. 2023;28:7433. <https://doi.org/10.3390/molecules28217433>.
135. Muller E, Bringel F, Vuilleumier S. Dichloromethane-degrading bacteria in the genomic age. *Res Microbiol*. 2011;162:869–76. <https://doi.org/10.1016/j.resmic.2011.01.008>.
136. Chen X, Köllner TG, Jia Q, Norris A, Santhanam B, Rabe P, Dickschat JS, Shaulsky G, Gershenzon J, Chen F. Terpene synthase genes in eukaryotes beyond plants and fungi: occurrence in social amoebae. *PNAS*. 2016;113:12132–7. <https://doi.org/10.1073/pnas.1610379113>.
137. Wilhelm RC. Following the terrestrial tracks of *Caulobacter*—redefining the ecology of a reputed aquatic oligotroph. *ISME J*. 2018;12:3025–37. <https://doi.org/10.1038/s41396-018-0257-z>.
138. Poindexter JS. Biological properties and classification of the *Caulobacter* group. *Bacteriol Rev*. 1964;28:231. <https://doi.org/10.1128/br.28.3.231-295.1964>.
139. Corgié S, Beguiristain T, Leyval C. Spatial distribution of bacterial communities and phenanthrene degradation in the rhizosphere of *Lolium perenne* L. *Appl Environ Microbiol*. 2004;70:3552–7. <https://doi.org/10.1128/AEM.70.6.3552-3557.2004>.
140. Lolium perenne L. *Appl Environ Microbiol*. 2004;70:3552–7. <https://doi.org/10.1128/AEM.70.6.3552-3557.2004>.
141. Yergeau E, Sanschagrin S, Beaumier D, Greer CW. Metagenomic analysis of the bioremediation of diesel-contaminated Canadian high arctic soils. *PLoS ONE*. 2012;7: e30058. <https://doi.org/10.1371/journal.pone.0030058>.
142. Chen T, Qu N, Wang J, Liu Y, Feng J, Zhang S, Xu C, Cao Z, Pan J, Li C. Effects of different ecological restoration methods on the soil bacterial community structure of a light rare earth tailings pond. *Plant Soil*. 2024;497:43–59. <https://doi.org/10.1007/s11104-023-06295-x>.
143. Yamada Y, Kuzuyama T, Komatsu M, Takagi M, Shin-ya K, Omura S. Terpene synthases are widely distributed in bacteria. *PNAS*. 2014;112:857–62. <https://doi.org/10.1073/pnas.1422108112>.
144. Koh SC, Park YI, Koo YM, So JS. Plant terpenes and lignin as natural cosubstrates in biodegradation of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). *Biotechnol Bioeng*. 2000;5:164–8. <https://doi.org/10.1007/BF02936588>.
145. Brown JM, Thompson IP, Paton GI, Singer AC. Enhanced biotransformation of TCE using plant terpenoids in contaminated groundwater. *Lett Appl Microbiol*. 2009;49:769–74. <https://doi.org/10.1111/j.1472-765X.2009.02738.x>.
146. McLoughlin E, Rhodes AH, Owen SM, Semple KT. Biogenic volatile organic compounds as a potential stimulator for organic contaminant degradation by soil microorganisms. *Environ Poll*. 2009;2009(157):86–94. <https://doi.org/10.1016/j.envpol.2008.07.029>.
147. Rudolf JD, Alsup TA, Xu B, Li Z. Bacterial terpenome. *Nat Prod Rep*. 2021;38:905–80. <https://doi.org/10.1039/d0np00066c>.
148. van Scholl L, Kuyper TW, Smits MM, Landeweert R, Hoffland E, Breemen N. Rock-eating mycorrhizas: Their role in plant nutrition and biogeochemical cycles. *Plant Soil*. 2008;303:35–47. <https://doi.org/10.1007/s11104-007-9513-0>.
149. Uroz S, Oger P, Lepleux C, Collignon C, Frey-Klett P, Turpault MP. Bacterial weathering and its contribution to nutrient cycling in temperate forest ecosystems. *Res Microbiol*. 2011;162:820–31. <https://doi.org/10.1016/j.resmic.2011.01.013>.

150. Adeleke R, Nwangburuka C, Oboirien B. Origins, roles and fate of organic acids in soils: a review. *S Afr J Bot.* 2017;108:393–406. <https://doi.org/10.1016/j.sajb.2016.09.002>.
151. Prabhu N, Borkar S, Garg S. Phosphate solubilization by microorganisms: overview, mechanisms, applications and advances. *Adv Biol Sci Res.* 2019. <https://doi.org/10.1016/B978-0-12-817497-5.00011-2>.
152. Tiwari S, Prasad V, Lata C. *Bacillus*: Plant growth promoting bacteria for sustainable agriculture and environment. In: *New and future developments in microbial biotechnology and bioengineering*. Elsevier; 2019. p. 43–55.
153. Cozzolino V, Monda H, Savy D, Di Meo V, Vinci G, Smalla K. Cooperation among phosphate-solubilizing bacteria, humic acids and arbuscular mycorrhizal fungi induces soil microbiome shifts and enhances plant nutrient uptake. *Chem Biol Techn Agric.* 2021;8:1–18. <https://doi.org/10.1186/s40538-021-00230-x>.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Conclusions of this Thesis

In this Thesis I focused on the role of microorganisms in improving plants resistance to abiotic stresses.

The use of bio-based solutions in arid and semi-arid environments

Agricultural production now more than ever needs sustainable approaches able to maintain environmental health and to improve crops productivity to meet human population needs. The study of drought-sensitive crops (**Chapter I and II**) highlights how microbial-based solutions are able to boost crop productivity under drought conditions, underlying the importance of these new technologies in a climate change scenario. To fully evaluate the impact on plant growth, field experiments are needed to understand how soil parameters, climatic conditions and natural soil microorganisms may interact with the microbial inoculant (Li C. et al., 2024; Custódio et al., 2022; Juyal et al., 2021; Pettersson and Bååth, 2013). The role of AMF in enhancing crop productivity has been deeply discussed by the scientific community (Berruti et al., 2016), as well as the use of PGPB as bio-based solutions (Chieb and Gachomo, 2023).

Chapter I focused on the role of AMF inoculation in improving pepper plant growth, enhancing both eco-physiological parameters, as well as qualitative and quantitative traits. Additionally, this study demonstrated that, under AMF inoculation, drought-sensitive crops as pepper are able to optimally grow under water limited conditions. This last result is particularly important in addressing climate change adaptation. In fact, the application of a reduced irrigation system allows agricultural production to better adapt to arid and semi-arid conditions.

Chapter II demonstrated that PGPB-based inoculants can be effective in strengthening plant growth in arid and semi-arid environments. In particular, this study demonstrated how pea plant productivity was positively impacted by the inoculation with native bacterial strains selected from the same geographic location in Tunisia. This result underlines how natural microbial resources might be exploited to boost agricultural productivity.

The importance of specific microorganism-microorganism interaction in improving plant growth

Considering the specificity that occurs in plant-microorganism interactions (van der Putten et al., 2013), field experiments are fundamental in addressing the comprehension of the complex relationships among the environment, the plant, the inoculum and the natural soil-borne microorganisms. Exploring the combined effect of the native microorganisms with the inoculum may allow

exploiting natural microbial resources to boost crop productivity (**Chapter I** and **II**).

Chapter I highlighted that AMF-based inoculation triggers specific associations between pepper plants and natural soil-borne microorganisms, particularly in the recruitment of potential PGPB.

Chapter II further strengthened this hypothesis, showing that PGPB inoculation was associated with the presence of natural soil-borne AMF and different natural PGPB. In particular, the correlation between AMF and bacteria highlighted the presence of PGPB previously recognized as mycorrhizal helper bacteria belonging to the so-called mycorrhizosphere (Agnolucci et al., 2019; Caddell et al., 2020; Giovannini et al., 2020). This result is particularly important in suggesting tailored inoculation approaches as effective in improving plant growth and productivity by exploiting the synergistic effects of soil microbial interactions.

The role of legumes as a natural source of nitrogen in sustainable agriculture

Legumes are commonly used in agriculture as a natural source of nitrogen in the soil (Brescia et al., 2023b, 2023a; Gritli et al., 2023; Zampieri et al., 2025).

Chapter II focused on pea to address the relevant need in improving legume production, suggesting also that a major role in boosting pea production is related to the cultivar choice. In fact, two different pea cultivars were used and one showed better production under field conditions. This result strengthens the need to select the adequate variety to better perform in field. Additionally, each variety was associated with specific putative PGPB, therefore a role in boosting the performance of one of the two varieties may have depended also on the specific microbial associations. This again underlines the importance of field experiments to evaluate which conditions mainly affect plant growth and to adapt the cultivation systems to the field.

Legumes are often used in intercropping systems, thanks to their ability to provide nitrogen to the intercropped plants, particularly with the ones that strongly rely on nitrogen chemical fertilization such as *B. napus* (Emery et al., 2021).

Chapter III was dedicated to how different legumes may affect *B. napus* growth and productivity. Canola is sensitive to drought (Elferjani and Soolanayakanahally, 2018) and the study was conducted in a semi-arid region to understand if a specific legume association and the relative shaping of the canola-associated bacteria may have improved its resistance to water deficit conditions. This study demonstrated that *M. sativa* intercropped with canola, despite being the most commonly used legume in intercropping systems (Kulkarni et al., 2018), showed lower canola growth and production parameters compared to the intercropping with *H. coronarium*, a less-adopted forage legume. Each legume-association was characterized by specific bacterial recruitment, linking the presence

of putative PGPB to a better canola performance. These results improved the knowledge on the different types of legumes that can be used in intercropping, showing that the most used forage legumes in intercropping are not always the correct choice as it depends on the specific field conditions.

Innovative approaches in soil microbial composition evaluation and monitoring

Considering the current climate change scenario and the growing frequency of land degradation phenomena (Huang et al., 2020), finding new methods to evaluate soil health is becoming fundamental. In my thesis we explored how bio-based solutions improve soil health and promote plant growth, especially in drought-prone environments.

The analysis of soil microbial communities proved to be an effective tool in understanding the complex dynamics between plants and microorganisms (Djemiel et al., 2022). Therefore, a specific microbial composition may be linked to a positive effect on plants. Additionally, the presence of particular taxa may give information on soil health status (Schloter et al., 2018).

Soil is a reservoir of molecules derived from different activities. Among the different molecules that can be found, VOCs represent a huge source of different compounds, either from biological sources (BVOCs) or anthropogenic activities (AVOCs) (Mangotra and Singh, 2024). Linking VOCs and soil microbiome composition provides insights on the ability of specific taxa to degrade toxic compounds, as well as to produce metabolites able to improve soil nutrients status (Ali et al., 2025). In this context, **Chapter IV** explored the tailored approach of linking VOC spectrum to microbiome composition in urban soils. Results highlighted that AMF inoculation improved soil health restoration, especially when combined with the use of cover plants.

This thesis results are strongly related to the use of metabarcoding analysis. Among the different omics techniques, metabarcoding is a promising tool to obtain an overview of the microbial composition in a certain condition (Joos et al., 2020). **Chapter I, II, III** demonstrated how metabarcoding can be used to investigate plant-microbial interactions, while **Chapter IV** provides an innovative use of this technique in addressing soil health evaluation. These aspects need to be now more than ever considered to successfully adapt agricultural production to climate change scenario and to reduce the increasing land degradation processes.

Final conclusions

The results presented in this Thesis highlight several fundamental research points in plant-microbiome interactions. Across all Chapters, a major point is played by symbiont-host compatibility between the variable response of different plant species and cultivars and the

inoculated microbes, whereas AMF or PGPB. This point underlines the importance of genotype-dependent microbial association and suggests that plant genotype plays a decisive role in modulating the efficiency of microbial inoculation. Differences observed across diverse field environments further underline the need to investigate combined genotype–environment (G×E) interactions, particularly in relation to microbiome composition and plant stress responses in arid and semi-arid environments. Another key research direction is the role of native microbial communities and their interactions with introduced inoculants, as well as the consequences of these interactions on plant growth. Both AM fungal- and PGPB-based solutions influence the recruitment of native soil microorganisms, underlining the central role of rhizosphere signaling and plant-mediated microbial selection in driving the composition of root-associated communities. These processes extend to the mycorrhizosphere and hyphosphere, where AMF appear to modulate bacterial colonization patterns and potentially interact with naturally occurring mycorrhizal helper bacteria. The findings from the field experiments additionally raise questions about the dynamics occurring between indigenous and introduced AM fungal strains, as well as the ecological mechanisms that determine whether inoculated microorganisms can efficiently colonize soil and roots or are replaced by native communities. Together, these aspects demonstrate that microbial inoculants play a central role in the complex tripartite plant-microbe-environment networks and that understanding how this network is structured and regulated is essential to implement more efficient and sustainable bio-based agricultural strategies able to meet the changing environmental conditions.

In conclusion, even though there is a current deep scientific knowledge in the use of beneficial soil microorganisms to boost agricultural productivity, the continuous variations in the environmental conditions require constant studies to fully understand their effects on plants. Additionally, climate change further exacerbates the manifestation of these variations, as well as their impact on agricultural productions. Therefore, new sustainable approaches able to mitigate climate change scenario and able to adapt agricultural production to the unexpected environmental constraints are needed.

References

- Abis, L., Loubet, B., Ciuraru, R., Lafouge, F., Houot, S., Nowak, V., Tripied, J., Dequiedt, S., Maron, P.A., Sadet-Bourgeteau, S., 2020. Reduced microbial diversity induces larger volatile organic compound emissions from soils. *Sci. Rep.* 10, 6104. <https://doi.org/10.1038/s41598-020-63091-8>
- Aboukota, M.E.S., Hassaballa, H., Elhini, M., Ganzour, S.K., 2024. Land degradation, desertification & environmental sensitivity to climate change in Alexandria and Beheira, Egypt. *Egypt. J. Soil Sci.* 64, 167–180. <https://doi.org/10.21608/ejss.2023.237386.1664>
- Adesina, O.S., Whitfield, S., Sallu, S.M., Sait, S.M., Pittchar, J., 2023. Bridging the gap in agricultural innovation research: a systematic review of push–pull biocontrol technology in sub-Saharan Africa. *Int. J. Agricult. Sustain.* 21, 2232696. <https://doi.org/10.1080/14735903.2023.2232696>
- Affortit, P., Ahmed, M.A., Grondin, A., Delzon, S., Carminati, A., Laplaze, L., 2024. Keep in touch: the soil–root hydraulic continuum and its role in drought resistance in crops. *J. Exp. Bot.* 75, 584–593. <https://doi.org/10.1093/jxb/erad312>
- Agnolucci, M., Palla, M., Cristani, C., Cavallo, N., Giovannetti, M., De Angelis, M., Gobbetti, M., Minervini, F., 2019. Beneficial plant microorganisms affect the endophytic bacterial communities of durum wheat roots as detected by different molecular approaches. *Front. Microbiol.* 10. <https://doi.org/10.3389/fmicb.2019.02500>
- Agrimonti, C., Lauro, M., Visioli, G., 2021. Smart agriculture for food quality: Facing climate change in the 21st century. *Crit. Rev. Food Sci. Nutr.* 61, 971–981. <https://doi.org/10.1080/10408398.2020.1749555>
- Ahluwalia, O., Singh, P.C., Bhatia, R., 2021. A review on drought stress in plants: Implications, mitigation and the role of plant growth promoting rhizobacteria. *Resour. Environ. Sustain.* 5, 100032. <https://doi.org/10.1016/j.resenv.2021.100032>
- Ahmad, I., Pichtel, J., Hayat, S. (A c. Di)., 2008. Plant-Bacteria interactions: Strategies and techniques to promote plant growth (1st ed.). Wiley. <https://doi.org/10.1002/9783527621989>
- Ali, Q., Khan, A.R., Raza, W., Bilal, M.S., Khalid, S., Ayaz, M., Khan, A.-U.-R., Mundra, S., 2025. Mechanisms of microbial VOC-mediated communication in plant ecosystems and agricultural applications. *J. Sustain. Agric. Environ.* 4, e70044. <https://doi.org/10.1002/sae2.70044>
- Ali, S., Tyagi, A., Park, S., Mir, R.A., Mushtaq, M., Bhat, B., Mahmoudi, H., Bae, H., 2022. Deciphering the plant microbiome to improve drought tolerance: Mechanisms and perspectives. *Environ. Exp. Bot.* 201, 104933. <https://doi.org/10.1016/j.envexpbot.2022.104933>
- Almeida, A., Mitchell, A.L., Tarkowska, A., Finn, R.D., 2018. Benchmarking taxonomic assignments based on 16S rRNA gene profiling of the microbiota from commonly sampled environments. *Gigascience* 7, giy054. <https://doi.org/10.1093/gigascience/giy054>
- Andrade, G., Linderman, R.G., Bethlenfalvay, G.J., 1998. Bacterial associations with the mycorrhizosphere and hyphosphere of the arbuscular mycorrhizal fungus *Glomus mosseae*. *Plant Soil* 202, 79–87. <https://doi.org/10.1023/A:1004397222241>
- Arcidiacono, M., Ercoli, L., Piazza, G., Pellegrino, E., 2025. Field inoculation with a local arbuscular mycorrhizal (AM) fungal consortium promotes sunflower agronomic traits without changing the composition of AM fungi coexisting inside the crop roots. *Appl. Soil Ecol.* 206, 105830. <https://doi.org/10.1016/j.apsoil.2024.105830>
- Asensio, D., Rapparini, F., Peñuelas, J., 2012. AM fungi root colonization increases the production of essential isoprenoids vs. nonessential isoprenoids especially under drought stress conditions or after jasmonic acid application. *Phytochemistry* 77, 149–161. <https://doi.org/10.1016/j.phytochem.2011.12.012>
- Augé, R.M., 2001. Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. *Mycorrhiza* 11, 3–42. <https://doi.org/10.1007/s005720100097>

- Augé, R.M., 2004. Arbuscular mycorrhizae and soil/plant water relations. *Can. J. Soil Sci.* 84, 373–381. <https://doi.org/10.4141/S04-002>
- Augé, R.M., Toler, H.D., Saxton, A.M., 2015. Arbuscular mycorrhizal symbiosis alters stomatal conductance of host plants more under drought than under amply watered conditions: a meta-analysis. *Mycorrhiza* 25, 13–24. <https://doi.org/10.1007/s00572-014-0585-4>
- Autio, A., Johansson, T., Motaroki, L., Minoia, P., Pellikka, P., 2021. Constraints for adopting climate-smart agricultural practices among smallholder farmers in Southeast Kenya. *Agric. Syst.* 194, 103284. <https://doi.org/10.1016/j.agsy.2021.103284>
- Azcón-Aguilar, C., Jaizme-Vega, M. C., Calvet, C., 2002. The contribution of arbuscular mycorrhizal fungi to the control of soil-borne plant pathogens. In S. Gianinazzi, H. Schüepp, J. M. Barea, & K. Haselwandter (A c. Di), *Mycorrhizal Technology in Agriculture* 187–197. Birkhäuser Basel. https://doi.org/10.1007/978-3-0348-8117-3_15
- Bağdat, E.Ş., Kahraman Ilikkan, Ö., Aydoğdu, E.Ö.A., Kimiran, A., Güngör, N.D., 2025. Microbial Production of Pyrazines (Nutty and Roasted Flavors), in: *Microbial Production of Food Bioactive Compounds*. Springer, Cham, pp. 885–915. https://doi.org/10.1007/978-3-031-90974-0_57
- Bahadur, A., Batool, A., Nasir, F., Jiang, S., Mingsen, Q., Zhang, Q., Pan, J., Liu, Y., Feng, H., 2019. Mechanistic insights into arbuscular mycorrhizal fungi-mediated drought stress tolerance in plants. *Int. J. Mol. Sci.* 20, 4199. <https://doi.org/10.3390/ijms20174199>
- Balasubramaniam, T., Shen, G., Esmacili, N., Zhang, H., 2023. Plants' response mechanisms to salinity stress. *Plants* 12, 2253. <https://doi.org/10.3390/plants12122253>
- Balestrini, R., Sillo, F., Boussageon, R., Wipf, D., Courty, P.E., 2024. The hidden side of interaction: microbes and roots get together to improve plant resilience. *J. Plant Interact.* 19, 2323991. <https://doi.org/10.1080/17429145.2024.2323991>
- Baloch, F.B., Zeng, N., Gong, H., Zhang, Z., Zhang, N., Baloch, S.B., Ali, S., Li, B., 2024. Rhizobacterial volatile organic compounds: Implications for agricultural ecosystems' nutrient cycling and soil health. *Heliyon* 10. <https://doi.org/10.1016/j.heliyon.2024.e40522>
- Banerjee, S., and van der Heijden, M.G.A., 2023. Soil microbiomes and one health. *Nat. Rev. Microbiol.* 21, 6–20. <https://doi.org/10.1038/s41579-022-00779-w>
- Bashan, Y., and Holguin, G., 1998. Proposal for the division of plant growth-promoting rhizobacteria into two classifications: Biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. *Soil Biol. Biochem.* 30, 1225–1228. [https://doi.org/10.1016/S0038-0717\(97\)00187-9](https://doi.org/10.1016/S0038-0717(97)00187-9)
- Bashir, S.S., Hussain, A., Hussain, S.J., Wani, O.A., Zahid Nabi, S., Dar, N.A., Baloch, F.S., Mansoor, S., 2021. Plant drought stress tolerance: understanding its physiological, biochemical and molecular mechanisms. *Biotechnol. Equip.* 35, 1912–1925. <https://doi.org/10.1080/13102818.2021.2020161>
- Battini, F., Grønlund, M., Agnolucci, M., Giovannetti, M., Jakobsen, I., 2017. Facilitation of phosphorus uptake in maize plants by mycorrhizosphere bacteria. *Sci. Rep.* 7, 4686. <https://doi.org/10.1038/s41598-017-04959-0>
- Berendsen R.L., Pieterse C.M., Bakker P.A., 2012. The rhizosphere microbiome and plant health. *Trends Plant Sci.* 17, 478–486. <https://doi.org/10.1016/j.tplan ts.2012.04.001>
- Berruti, A., Lumini, E., Balestrini, R., Bianciotto, V., 2016. Arbuscular mycorrhizal fungi as natural biofertilizers: Let's benefit from past successes. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.01559>
- Bharadwaj, D.P., Alström, S., Lundquist, P.-O., 2012. Interactions among *Glomus irregulare*, arbuscular mycorrhizal spore-associated bacteria, and plant pathogens under in vitro conditions. *Mycorrhiza* 22, 437–447. <https://doi.org/10.1007/s00572-011-0418-7>
- Bittelli, M., 2010. Measuring Soil Water Potential for Water Management in Agriculture: A Review. *Sustainability* 2, 1226–1251. <https://doi.org/10.3390/su2051226>
- Bleidorn, C., 2016. Third generation sequencing: technology and its potential impact on evolutionary biodiversity research. *Syst. Biodivers.* 14, 1–8. <https://doi.org/10.1080/14772000.2015.1099575>

- Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolk, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Priesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Bonfante, P., and Genre, A., 2008. Plants and arbuscular mycorrhizal fungi: An evolutionary-developmental perspective. *Trends Plant Sci.* 13, 492–498. <https://doi.org/10.1016/j.tplants.2008.07.001>
- Bonfante, P., and Genre, A., 2010. Mechanisms underlying beneficial plant–fungus interactions in mycorrhizal symbiosis. *Nat. Commun.* 1, 48. <https://doi.org/10.1038/ncomms1046>
- Boutsika, A., Xanthopoulou, A., Tanou, G., Zacharatu, M., –Evangelia, Vernikos, M., Nianiou-Obeidat, I., Ganopoulos, I., Mellidou, I., 2024. A microbiome survey of contrasting potato terroirs using 16S rRNA long-read sequencing. *Plant Soil* 505, 431–448. <https://doi.org/10.1007/s11104-024-06686-8>
- Boyno, G. and Demir, S., 2022. Plant-mycorrhiza communication and mycorrhizae in inter-plant communication. *Symbiosis* 86, 155–168. <https://doi.org/10.1007/s13199-022-00837-0>
- Branch, H.A., Moxley, D.R., Angert, A.L., 2024. Regional differences in leaf evolution facilitate photosynthesis following severe drought. *New Phytol.* 243, 2457–2469. <https://doi.org/10.1111/nph.19963>
- Brescia, F., Sillo, F., Balestrini, R., Sbrana, C., Zampieri, E., 2023a. Characterization of endophytic bacteria isolated from root nodules of lentil in intercropping with durum wheat. *Curr. Res. Microb. Sci.* 5, 100205. <https://doi.org/10.1016/j.crmicr.2023.100205>
- Brescia, F., Sillo, F., Franchi, E., Pietrini, I., Montesano, V., Marino, G., Haworth, M., Zampieri, E., Fusini, D., Schillaci, M., Papa, R., Santamarina, C., Vita, F., Chitarra, W., Nerva, L., Petruzzelli, G., Mennone, C., Centritto, M., Balestrini, R., 2023b. The ‘microbiome counterattack’: Insights on the soil and root-associated microbiome in diverse chickpea and lentil genotypes after an erratic rainfall event. *Environ. Microbiol. Rep.* 15, 459–483. <https://doi.org/10.1111/1758-2229.13167>
- Burgess, A.J., Correa Cano, M.E., Parkes, B., 2022. The deployment of intercropping and agroforestry as adaptation to climate change. *Crop Environ.* 1, 145–160. <https://doi.org/10.1016/j.crope.2022.05.001>
- Burroughs, W.J., 2007. Climate Change: A Multidisciplinary Approach. Cambridge University Press.
- Caddell, D., Louie, K., Bowen, B., Sievert, J., Hollingsworth, J., Dahlberg, J., Purdom, E., Northen, T., Coleman-Derr, D., 2020. Drought shifts sorghum root metabolite and microbiome profiles and enriches the stress response factor pipecolic acid. *Phytobiomes J.* 7:4, 449–463. <https://doi.org/10.1101/2020.11.08.373399>
- Callahan, B.J., McMurdie, P.J., Holmes, S.P., 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *ISME J.* 11, 2639–2643. <https://doi.org/10.1038/ismej.2017.119>
- Cardoso, E.J.B.N., Vasconcellos, R.L.F., Bini, D., Miyauchi, M.Y.H., Santos, C.A. dos, Alves, P.R.L., Paula, A.M. de, Nakatani, A.S., Pereira, J. de M., Nogueira, M.A., 2013. Soil health: looking for suitable indicators. What should be considered to assess the effects of use and management on soil health? *Sci. Agric. (Piracicaba, Braz.)* 70, 274–289. <https://doi.org/10.1590/S0103-90162013000400009>
- Carminati, A., Javaux, M., 2020. Soil Rather Than Xylem Vulnerability Controls Stomatal Response to Drought. *Trends Plant Sci.* 25, 868–880. <https://doi.org/10.1016/j.tplants.2020.04.003>

- Cassol, I., Ibañez, M., Bustamante, J.P., 2025. Key features and guidelines for the application of microbial alpha diversity metrics. *Sci. Rep* 15, 622. <https://doi.org/10.1038/s41598-024-77864-y>
- Chandrasekaran, M., Paramasivan, M., Sahayarayan, J.J., 2022. Microbial volatile organic compounds: An alternative for chemical fertilizers in sustainable agriculture development. *Microorganisms* 11, 42. <https://doi.org/10.3390/microorganisms11010042>
- Chen, H. and Jiang, J.-G., 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environ. Rev.* 18, 309–319. <https://doi.org/10.1139/A10-014>
- Chen, H., Bullock, D.A., Alonso, J.M., Stepanova, A.N., 2021. To Fight or to Grow: The Balancing Role of Ethylene in Plant Abiotic Stress Responses. *Plants (Basel)* 11, 33. <https://doi.org/10.3390/plants11010033>
- Chen, M., Arato, M., Borghi, L., Nouri, E., Reinhardt, D., 2018. Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application. *Front. Plant Sci.* 9. <https://doi.org/10.3389/fpls.2018.01270>
- Chieb, M., and Gachomo, E.W., 2023. The role of plant growth promoting rhizobacteria in plant drought stress responses. *BMC Plant Biology* 23, 407. <https://doi.org/10.1186/s12870-023-04403-8>
- Chitarra, W., Pagliarani, C., Maserti, B., Lumini, E., Siciliano, I., Cascone, P., Schubert, A., Gambino, G., Balestrini, R., Guerrieri, E., 2016. Insights on the impact of arbuscular mycorrhizal symbiosis on tomato tolerance to water stress. *Plant Physiol.* 171, 1009–1023. <https://doi.org/10.1104/pp.16.00307>
- Chiu, C.H. and Paszkowski, U., 2019. Mechanisms and impact of symbiotic phosphate acquisition. *Cold Spring Harb. Perspect. Biol.* 11, a034603. <https://doi.org/10.1101/cshperspect.a034603>
- Choi, Y.-W., and Eltahir, E.A.B., 2023. Near-term climate change impacts on food crops productivity in East Africa. *Theor. Appl. Climatol.* 152, 843–860. <https://doi.org/10.1007/s00704-023-04408>
- Choi, Y.-W., Campbell, D.J., Eltahir, E.A.B., 2023. Near-term regional climate change in East Africa. *Clim. Dyn.* 61, 961–978. <https://doi.org/10.1007/s00382-022-06591-9>
- Choudhary, A., Kumar, A., Kaur, N., 2020. ROS and oxidative burst: Roots in plant development. *Plant Divers.* 42, 33–43. <https://doi.org/10.1016/j.pld.2019.10.002>
- Clarke, J., Wu, H.-C., Jayasinghe, L., Patel, A., Reid, S., Bayley, H., 2009. Continuous base identification for single-molecule nanopore DNA sequencing. *Nature Nanotech.* 4, 265–270. <https://doi.org/10.1038/nnano.2009.12>
- Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. <https://doi.org/10.1093/nar/gkt1244>
- Cotula, L., Toulmin, C., Hesse, C., 2004. Tenure and Administration in Africa: Lessons of Experience and Emerging Issues. London: International Institute for Environment and Development (IIED), Drylands Programme, 2004. ISBN 1-84369-497-2.
- Custódio, V., Gonin, M., Stabl, G., Bakhoun, N., Oliveira, M.M., Gutjahr, C., Castrillo, G., 2022. Sculpting the soil microbiota. *Plant J.* 109, 508–522. <https://doi.org/10.1111/tpj.15568>
- Dai, A., 2011. Drought under global warming: a review. *WIREs Climate Change* 2, 45–65. <https://doi.org/10.1002/wcc.81>
- David, B.V., Chandrasehar, G., Selvam, P.N., 2018. *Pseudomonas fluorescens*: A plant-growth-promoting rhizobacterium (PGPR) with potential role in biocontrol of pests of crops, in: Prasad, R., Gill, S.S., Tuteja, N. (Eds.), Crop Improvement Through Microbial Biotechnology. Elsevier, pp. 221–243. <https://doi.org/10.1016/B978-0-444-63987-5.00010-4>
- de Andrade, L.A., Santos, C.H.B., Frezarin, E.T., Sales, L.R., Rigobelo, E.C., 2023. Plant growth-promoting rhizobacteria for sustainable agricultural production. *Microorganisms* 11, 1088. <https://doi.org/10.3390/microorganisms11041088>
- Deel, H.L., Moore, J.M., Manter, D.K., 2024. SEMWISE: A national soil health scoring framework for agricultural systems. *Appl. Soil Ecol.* 195, 105273. <https://doi.org/10.1016/j.apsoil.2024.105273>

- Delaporte-Quintana, P., Lovaisa, N.C., Rapisarda, V.A., Pedraza, R.O., 2020. The plant growth promoting bacteria *Gluconacetobacter diazotrophicus* and *Azospirillum brasilense* contribute to the iron nutrition of strawberry plants through siderophores production. *Plant. Growth. Regul.* 91, 185–199. <https://doi.org/10.1007/s10725-020-00598-0>
- DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72, 5069–5072. <https://doi.org/10.1128/AEM.03006-05>
- Diop, M., Chirinda, N., Beniaich, A., El Gharous, M., El Mejahed, K., 2022. Soil and water conservation in Africa: State of play and potential role in tackling soil degradation and building soil health in agricultural lands. *Sustainability* 14, 13425. <https://doi.org/10.3390/su142013425>
- Djemiel, C., Dequiedt, S., Karimi, B., Cottin, A., Horrigue, W., Bailly, A., Boutaleb, A., Sadet-Bourgeteau, S., Maron, P.-A., Chemidlin Prévost-Bouré, N., Ranjard, L., Terrat, S., 2022. Potential of meta-omics to provide modern microbial indicators for monitoring soil quality and securing food production. *Front. Microbiol.* 13. <https://doi.org/10.3389/fmicb.2022.889788>
- Dowling, A., Sadras, V., Roberts, P., Doolette, A., Zhou, Y., Denton, M., 2021. Legume-oilseed intercropping in mechanised broadacre agriculture – a review. *Field Crops Res.* 260, 107980. <https://doi.org/10.1016/j.fcr.2020.107980>
- Eid, J., Fehr, A., Gray, J., Luong, K., Lyle, J., Otto, G., Peluso, P., Rank, D., Baybayan, P., Bettman, B., Bibillo, A., Bjornson, K., Chaudhuri, B., Christians, F., Cicero, R., Clark, S., Dalal, R., deWinter, A., Dixon, J., Foquet, M., Gaertner, A., Hardenbol, P., Heiner, C., Hester, K., Holden, D., Kearns, G., Kong, X., Kuse, R., Lacroix, Y., Lin, S., Lundquist, P., Ma, C., Marks, P., Maxham, M., Murphy, D., Park, I., Pham, T., Phillips, M., Roy, J., Sebra, R., Shen, G., Sorenson, J., Tomaney, A., Travers, K., Trulson, M., Vieceli, J., Wegener, J., Wu, D., Yang, A., Zaccarin, D., Zhao, P., Zhong, F., Korlach, J., Turner, S., 2009. Real-Time DNA Sequencing from Single Polymerase Molecules. *Science* 323, 133–138. <https://doi.org/10.1126/science.1162986>
- Elferjani, R., and Soolanayakanahally, R., 2018. Canola responses to drought, heat, and combined stress: Shared and specific effects on carbon assimilation, seed yield, and oil composition. *Front. Plant Sci.* 9. <https://doi.org/10.3389/fpls.2018.01224>
- Emery, S.E., Anderson, P., Carlsson, G., Friberg, H., Larsson, M.C., Wallenhammar, A.-C., Lundin, O., 2021. The potential of intercropping for multifunctional crop protection in oilseed rape (*Brassica napus* L.). *Front. Agron.* 3. <https://doi.org/10.3389/fagro.2021.782686>
- Fadiji, A.E., Xiong, C., Egidi, E., Singh, B.K., 2024. Formulation challenges associated with microbial biofertilizers in sustainable agriculture and paths forward. *J. Sustain. Agric. Environ.* 3, e70006. <https://doi.org/10.1002/sae2.70006>
- Fageria, N.K., 2012. Role of Soil Organic Matter in Maintaining Sustainability of Cropping Systems. *Commun. Soil Sci. Plant Anal.* 43, 2063–2113. <https://doi.org/10.1080/00103624.2012.697234>
- Faghihinia, M., Jansa, J., Halverson, L.J., Staddon, P.L., 2023. Hyphosphere microbiome of arbuscular mycorrhizal fungi: a realm of unknowns. *Biol. Fertil. Soils* 59, 17–34. <https://doi.org/10.1007/s00374-022-01683-4>
- FAO (Ed.), 2006. Guidelines for soil description, 4. ed. ed. Food and Agriculture Organization of the United Nations, Rome.
- Fincheira, P., and Quiroz, A., 2026. Chapter 17 - Volatiles produced by soil bacteria as biocontrol and plant growth agents, Editor(s): Gustavo Santoyo, Soil Microorganisms for Plant Growth Promotion and Soil Health, Elsevier, pp. 399-419, ISBN 9780443340550, <https://doi.org/10.1016/B978-0-443-34055-0.00002-3>.
- Flexas, J., and Medrano, H., 2002. Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. *Ann. Bot.* 89, 183–189. <https://doi.org/10.1093/aob/mcf027>
- Francioli, D., Lentendu, G., Lewin, S., Kolb, S., 2021. DNA metabarcoding for the characterization of terrestrial microbiota—pitfalls and solutions. *Microorganisms* 9, 361. <https://doi.org/10.3390/microorganisms9020361>
- Frey-Klett, P., Garbaye, J., Tarkka, M., 2007. The mycorrhiza helper bacteria revisited. *New Phytol.* 176, 22–36. <https://doi.org/10.1111/j.1469-8137.2007.02191.x>
- Garbaye, J., 1994. Tansley Review No. 76 Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytol.* 128, 197–210. <https://doi.org/10.1111/j.1469-8137.1994.tb04003.x>

- Genre, A., Capitanio, S., Bonfante, P., 2024. Signals and host cell remodeling in arbuscular mycorrhizal symbiosis, in: Hsueh, Y.-P., Blackwell, M. (Eds.), *Fungal Associations*. Springer International Publishing, Cham, pp. 231–247. https://doi.org/10.1007/978-3-031-41648-4_9
- Gianinazzi, S., and Schüepp, H. (A c. Di)., 1994. Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhäuser Basel. <https://doi.org/10.1007/978-3-0348-8504-1>
- Giller, K.E., Corbeels, M., Nyamangara, J., Triomphe, B., Affholder, F., Scopel, E., Tittonell, P., 2011. A research agenda to explore the role of conservation agriculture in African smallholder farming systems. *Field Crops Res.* 124, 468–472. <https://doi.org/10.1016/j.fcr.2011.04.010>
- Giovannini, L., Palla, M., Agnolucci, M., Avio, L., Sbrana, C., Turrini, A., Giovannetti, M., 2020. Arbuscular mycorrhizal fungi and associated microbiota as plant biostimulants: research strategies for the selection of the best performing inocula. *Agronomy* 10, 106. <https://doi.org/10.3390/agronomy10010106>
- Goldberg-Yehuda, N., Nachshon, U., Assouline, S., Mau, Y., 2024. The effect of mechanical compaction on the soil water retention curve: Insights from a rapid image analysis of micro-CT scanning. *CATENA* 242, 108068. <https://doi.org/10.1016/j.catena.2024.108068>
- Goodwin, S., McPherson, J.D., McCombie, W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. *Nat. Rev. Genet.* 17, 333–351. <https://doi.org/10.1038/nrg.2016.49>
- Griffiths, B.S. and Philippot, L., 2013. Insights into the resistance and resilience of the soil microbial community. *FEMS Microbiol. Rev.* 37, 112–129. <https://doi.org/10.1111/j.1574-6976.2012.00343.x>
- Gritli, T., Ellouze, W., Wu, H., Taamali, W., Fu, W., Alfeddy, M.N., Ouahmane, L., Courty, P.E., Wipf, D., El Idrissi, M.M., Chen, B., Mnasri, B., 2023. Molecular characterization of arbuscular mycorrhizal communities associated with *Lathyrus cicera* L. grown in northern Tunisia soils. *Symbiosis* 90, 81–90. <https://doi.org/10.1007/s13199-023-00922-y>
- Gu, L., Chen, J., Yin, J., Sullivan, S.C., Wang, H.-M., Guo, S., Zhang, L., Kim, J.-S., 2020. Projected increases in magnitude and socioeconomic exposure of global droughts in 1.5 and 2°C warmer climates. *Hydrol. Earth Syst. Sci.* 24, 451–472. <https://doi.org/10.5194/hess-24-451-2020>
- Guillen-Otero, T., Lee, S., Chen, C., Szoevenyi, P., Kessler, M., 2023. A metabarcoding protocol targeting two DNA regions to analyze root-associated fungal communities in ferns and lycophytes. *Appl. Plant Sci.* 11, e11523. <https://doi.org/10.1002/aps3.11523>
- Guo, Q., Liu, L., Barkla, B.J., 2019. Membrane lipid remodeling in response to salinity. *Int. J. Mol. Sci.* 20, 4264. <https://doi.org/10.3390/ijms20174264>
- Hartmann, A., Weiler, M., Blume, T., 2020. The impact of landscape evolution on soil physics: evolution of soil physical and hydraulic properties along two chronosequences of proglacial moraines. *Earth Syst. Sci. Data* 12, 3189–3204. <https://doi.org/10.5194/essd-12-3189-2020>
- Hisdal, H., Tallaksen, L.M., Gauster, T., Bloomfield, J.P., Parry, S., Prudhomme, C., Wanders, N. (2024). Chapter 5 - Hydrological drought characteristics. In: Tallaksen, L.M., Van Lanen, H.A.J. (Eds.), *Hydrological Drought. Processes and Estimation Methods for Streamflow and Groundwater*. Developments in Water Science, 48, Elsevier Science B.V., pp. 139–198., *Hydrological Drought (Second Edition)*, Elsevier, 157-231, ISBN 9780128190821, <https://doi.org/10.1016/B978-0-12-819082-1.00006-0>.
- Huang, H., Ullah, F., Zhou, D.-X., Yi, M., Zhao, Y., 2019. Mechanisms of ROS regulation of plant development and stress responses. *Front. Plant Sci.* 10. <https://doi.org/10.3389/fpls.2019.00800>
- Huang, J., Zhang, G., Zhang, Y., Guan, X., Wei, Y., Guo, R., 2020. Global desertification vulnerability to climate change and human activities. *Land Degrad. Dev* 31, 1380–1391. <https://doi.org/10.1002/ldr.3556>
- Huang, X.-F., Chaparro, J.M., Reardon, K.F., Zhang, R., Shen, Q., Vivanco, J.M., 2014. Rhizosphere interactions: root exudates, microbes, and microbial communities. *Botany* 92, 267–275. <https://doi.org/10.1139/cjb-2013-0225>
- Hung, R., Lee, S., Bennett, J.W., 2013. *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. *Fungal Ecol.* 6, 19–26. <https://doi.org/10.1016/j.funeco.2012.09.005>

- Huss, C.P., Holmes, K.D., Blubaugh, C.K., 2022. Benefits and risks of intercropping for crop resilience and pest management. *J. Econ. Entomol.* 115, 1350–1362. <https://doi.org/10.1093/jee/toac045>
- Ilahi, H., Hsouna, J., Ellouze, W., Gritli, T., Chihoui, S., Barhoumi, F., Najib Elfeddy, M., Bachkouel, S., Ouahmane, L., Tambong, J.T., Mnasri, B., 2021. Phylogenetic study of rhizobia nodulating pea (*Pisum sativum*) isolated from different geographic locations in Tunisia. *Syst. Appl. Microbiol.* 44, 126221. <https://doi.org/10.1016/j.syapm.2021.126221>
- Ilahi, H., Zampieri, E., Sbrana, C., Brescia, F., Giovannini, L., Mahmoudi, R., Gohari, G., El Idrissi, M.M., Alfeddy, M.N., Schillaci, M., Ouahmane, L., Calvo, A., Sillo, F., Fotopoulos, V., Balestrini, R., Mnasri, B., 2024. Impact of two *Erwinia* sp. on the response of diverse *Pisum sativum* genotypes under salt stress. *Physiol. Mol. Biol. Plants* 30, 249–267. <https://doi.org/10.1007/s12298-024-01419-8>
- Insam, H., and Seewald, M.S.A., 2010. Volatile organic compounds (VOCs) in soils. *Biol. Fertil. Soils* 46, 199–213. <https://doi.org/10.1007/s00374-010-0442-3>
- Intana, W., Kheawlang, S., Sunpapao, A., 2021. *Trichoderma asperellum* T76-14 Released Volatile Organic Compounds against Postharvest Fruit Rot in Muskmelons (*Cucumis melo*) Caused by *Fusarium incarnatum*. *J. Fungi* 7, 46. <https://doi.org/10.3390/jof7010046>
- Intergovernmental Panel On Climate Change, 2022. Climate Change and Land: IPCC Special Report on Climate Change, Desertification, Land Degradation, Sustainable Land Management, Food Security, and Greenhouse Gas Fluxes in Terrestrial Ecosystems, 1st ed. Cambridge University Press. <https://doi.org/10.1017/9781009157988>
- International Fund for Agricultural Development (IFAD). *The Future of Agriculture in Sub-Saharan Africa*. Rome: IFAD, 2016. URL: https://www.ifad.org/documents/38714170/42030191/future_agriculture_sahara_e.pdf/1cb6b896-b9c1-0bb8-87b8-83df3153d0af
- Jha, C.K., Aeron, A., Patel, B.V., Maheshwari, D.K., Saraf, M., 2011. *Enterobacter*: Role in plant growth promotion, in: Maheshwari, D.K. (Ed.), *Bacteria in Agrobiolgy: Plant Growth Responses*. Springer, Berlin, Heidelberg, pp. 159–182. https://doi.org/10.1007/978-3-642-20332-9_8
- Jian, J., Du, X., Stewart, R.D., 2020. A database for global soil health assessment. *Sci. Data* 7, 16. <https://doi.org/10.1038/s41597-020-0356-3>
- Jin, Z., Jiang, F., Wang, L., Declerck, S., Feng, G., Zhang, L., 2024. Arbuscular mycorrhizal fungi and *Streptomyces*: brothers in arms to shape the structure and function of the hyphosphere microbiome in the early stage of interaction. *Microbiome* 12, 83. <https://doi.org/10.1186/s40168-024-01811-2>
- Joos, L., Beirinckx, S., Haegeman, A., Debode, J., Vandecasteele, B., Baeyen, S., Goormachtig, S., Clement, L., De Tender, C., 2020. Daring to be differential: metabarcoding analysis of soil and plant-related microbial communities using amplicon sequence variants and operational taxonomical units. *BMC Genomics* 21, 733. <https://doi.org/10.1186/s12864-020-07126-4>
- Juyal, A., Otten, W., Baveye, P.C., Eickhorst, T., 2021. Influence of soil structure on the spread of *Pseudomonas fluorescens* in soil at microscale. *Eur. J. Soil Sci.* 72, 141–153. <https://doi.org/10.1111/ejss.12975>
- Kakouridis, A., Hagen, J.A., Kan, M.P., Mambelli, S., Feldman, L.J., Herman, D.J., Weber, P.K., Pett-Ridge, J., Firestone, M.K., 2022. Routes to roots: direct evidence of water transport by arbuscular mycorrhizal fungi to host plants. *New Phytol.* 236, 210–221. <https://doi.org/10.1111/nph.18281>
- Kanchiswamy, C.N., Malnoy, M., Maffei, M.E., 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Front. Plant Sci.* 6. <https://doi.org/10.3389/fpls.2015.00151>
- Kangogo, D., Dentoni, D., Bijman, J., 2021. Adoption of climate-smart agriculture among smallholder farmers: Does farmer entrepreneurship matter? *Land Use Pol.* 109, 105666. <https://doi.org/10.1016/j.landusepol.2021.105666>
- Kelbessa, B.G., Dubey, M., Catara, V., Ghadamgahi, F., Ortiz, R., Vetukuri, R.R., 2023. Potential of plant growth-promoting rhizobacteria to improve crop productivity and adaptation to a changing climate. *CABI Reviews* 2023. <https://doi.org/10.1079/cabireviews.2023.0001>
- Kibblewhite, M.G., Ritz, K., Swift, M.J., 2007. Soil health in agricultural systems. *Phil. Trans. R. Soc. B.* 363, 685–701. <https://doi.org/10.1098/rstb.2007.2178>

- Kimutai, J., Barnes, C., Zachariah, M., Philip, S.Y., Kew, S.F., Pinto, I., Wolski, P., Koren, G., Vecchi, G., Yang, W., Li, S., Vahlberg, M., Singh, R., Heinrich, D., Arrighi, J., Marghidan, C.P., Thalheimer, L., Kane, C., Raju, E., Otto, F.E.L., 2025. Human-induced climate change increased 2021–2022 drought severity in horn of Africa. *Weather Clim. Extrem.* 47, 100745. <https://doi.org/10.1016/j.wace.2025.100745>
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41, e1. <https://doi.org/10.1093/nar/gks808>
- Koehler, T., Wankmüller, F.J.P., Sadok, W., Carminati, A., 2023. Transpiration response to soil drying versus increasing vapor pressure deficit in crops: physical and physiological mechanisms and key plant traits. *J. Exp. Bot.* 74, 4789–4807. <https://doi.org/10.1093/jxb/erad221>
- Kosmas, C., Ferrara, A., Briasouli, H., Imeson, A., 1999. Methodology for mapping environmentally sensitive areas (ESAs) to desertification. In C. Kosmas, M. Kirkby, & N. Geeson (Eds.), *The MEDALUS project: Mediterranean desertification and land use manual on key indicators of desertification and mapping environmentally sensitive areas to desertification*, pp. 31–47. Brussels: European Union.
- Kshetri, L., Naseem, F., Pandey, P., 2019. Role of *Serratia* sp. as biocontrol agent and plant growth stimulator, with prospects of biotic stress management in plant. *Microorganisms for Sustainability*, pp. 169–200. Springer, Singapore. https://doi.org/10.1007/978-981-13-6986-5_6
- Kuchikata, H., Sano, M., Fujiwara, F., Murashima, K., Kumaishi, K., Narukawa, M., Nose, Y., Kobayashi, M., Hamamoto, S., Kobayashi, N.I., Sugiyama, A., Nihei, N., Ichihashi, Y., Kusano, M., 2024. Soil volatilomics uncovers tight linkage between soybean presence and soil omics profiles in agricultural fields. *Sci. Rep.* 14, 20541. <https://doi.org/10.1038/s41598-024-70873-x>
- Kulkarni, K.P., Tayade, R., Asekova, S., Song, J.T., Shannon, J.G., Lee, J.-D., 2018. Harnessing the potential of forage legumes, alfalfa, soybean, and cowpea for sustainable agriculture and global food security. *Front. Plant Sci.* 9. <https://doi.org/10.3389/fpls.2018.01314>
- Kumawat, K. C., Razdan, N., Saharan, K., 2022. Rhizospheric microbiome: Bio-based emerging strategies for sustainable agriculture development and future perspectives. *Microbiol. Res.* 254, 126901. <https://doi.org/10.1016/j.micres.2021.126901>
- Kwaaitaal, M., Nielsen, M.E., Böhlenius, H., Thordal-Christensen, H., 2017. The plant membrane surrounding powdery mildew haustoria shares properties with the endoplasmic reticulum membrane. *J. Exp. Bot.* 68, 5731–5743. <https://doi.org/10.1093/jxb/erx403>
- Lakhiar, I.A., Yan, H., Zhang, C., Zhang, J., Wang, G., Deng, S., Syed, T.N., Wang, B., Zhou, R., 2024. A review of evapotranspiration estimation methods for climate-smart agriculture tools under a changing climate: vulnerabilities, consequences, and implications. *J. Water Clim. Change* 16, 249–288. <https://doi.org/10.2166/wcc.2024.048>
- Lanfranco, L., Bonfante, P., Genre, A., 2016. The mutualistic interaction between plants and arbuscular mycorrhizal fungi. *Microbiol. Spectr.* 4. <https://doi.org/10.1128/microbiolspec.funk-0012-2016>
- Laranjeira, S., Fernandes-Silva, A., Reis, S., Torcato, C., Raimundo, F., Ferreira, L., Carnide, V., Marques, G., 2021. Inoculation of plant growth promoting bacteria and arbuscular mycorrhizal fungi improve chickpea performance under water deficit conditions. *Appl. Soil Ecol.* 164, 103927. <https://doi.org/10.1016/j.apsoil.2021.103927>
- Lawrence, T.J., Vilbig, J.M., Kangogo, G., Fèvre, E.M., Deem, S.L., Gluecks, I., Sagan, V., Shacham, E., 2023. Spatial changes to climatic suitability and availability of agropastoral farming systems across Kenya (1980–2020). *Outlook Agric.* 52, 186–199. <https://doi.org/10.1177/00307270231176577>
- Lee, H.B., Jeong, D.H., Cho, B.C., Park, J.S., 2023. Comparative analyses of eight primer sets commonly used to target the bacterial 16S rRNA gene for marine metabarcoding-based studies. *Front. Mar. Sci.* 10. <https://doi.org/10.3389/fmars.2023.1199116>
- Leggieri, P.A., Liu, Y., Hayes, M., Connors, B., Seppälä, S., O'Malley, M.A., Venturelli, O.S., 2021. Integrating Systems and Synthetic Biology to Understand and Engineer Microbiomes. *Annu. Rev. Biomed. Eng.* 23, 169–201. <https://doi.org/10.1146/annurev-bioeng-082120-022836>

- Lehmann, J., and Kleber, M., 2015. The contentious nature of soil organic matter. *Nature* 528, 60–68. <https://doi.org/10.1038/nature16069>
- Lehmann, J., Bossio, D.A., Kögel-Knabner, I., Rillig, M.C., 2020. The concept and future prospects of soil health. *Nat. Rev. Earth Environ.* 1, 544–553. <https://doi.org/10.1038/s43017-020-0080-8>
- Lenoir, I., Fontaine, J., Lounès-Hadj Sahraoui, A., 2016. Arbuscular mycorrhizal fungal responses to abiotic stresses: A review. *Phytochemistry* 123, 4–15. <https://doi.org/10.1016/j.phytochem.2016.01.002>
- Li, C., Chen, X., Jia, Z., Zhai, L., Zhang, B., Grütters, U., Ma, S., Qian, J., Liu, X., Zhang, J., Müller, C., 2024. Meta-analysis reveals the effects of microbial inoculants on the biomass and diversity of soil microbial communities. *Nat. Ecol. Evol.* 8, 1270–1284. <https://doi.org/10.1038/s41559-024-02437-1>
- Li, M.-Y., Wang, W., Mo, F., Ren, A.-T., Wang, Z.-Y., Zhu, Y., Xiong, Y.-C., 2024. Seven-year long-term inoculation with *Funneliformis mosseae* increases maize yield and soil carbon storage evidenced by in situ ¹³C-labeling in a dryland. *Sci. Total Environ.* 944, 173975. <https://doi.org/10.1016/j.scitotenv.2024.173975>
- Lin, H., and Peddada, S.D., 2020. Analysis of compositions of microbiomes with bias correction. *Nat. Commun.* 11, 3514. <https://doi.org/10.1038/s41467-020-17041-7>
- Lipper, L., Thornton, P., Campbell, B.M., Baedeker, T., Braimoh, A., Bwalya, M., Caron, P., Cattaneo, A., Garrity, D., Henry, K., Hottle, R., Jackson, L., Jarvis, A., Kossam, F., Mann, W., McCarthy, N., Meybeck, A., Neufeldt, H., Remington, T., Sen, P.T., Sessa, R., Shula, R., Tibu, A., Torquebiau, E.F., 2014. Climate-smart agriculture for food security. *Nat. Clim. Chang.* 4, 1068–1072. <https://doi.org/10.1038/nclimate2437>
- Liu, C., Siri, M., Li, H., Ren, C., Huang, J., Feng, C., Liu, K., 2023. Drought is threatening plant growth and soil nutrients of grassland ecosystems: A meta-analysis. *Ecol. Evol.* 13, e10092. <https://doi.org/10.1002/ece3.10092>
- Liu, X., Le Roux, X., Salles, J.F., 2022. The legacy of microbial inoculants in agroecosystems and potential for tackling climate change challenges. *iScience* 25, 103821. <https://doi.org/10.1016/j.isci.2022.103821>
- Loreto, F., Dicke, M., Schnitzler, J.-P., Turlings, T.C.J., 2014. Plant volatiles and the environment. *Plant Cell Env.* 37, 1905–1908. <https://doi.org/10.1111/pce.12369>
- Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. <https://doi.org/10.1186/s13059-014-0550-8>
- Lu, Y., and Fricke, W., 2023. Salt Stress—Regulation of Root Water Uptake in a Whole-Plant and Diurnal Context. *Int. J. Mol. Sci.* 24, 8070. <https://doi.org/10.3390/ijms24098070>
- Lu, Y., Zhu, Y., Huang, L., Pu, Y., Sun, X., Feng, J., Sun, K., n.d. Antifungal mechanism of ketone volatile organic compounds against *Pseudogymnoascus destructans*. *Virulence* 16, 2569627. <https://doi.org/10.1080/21505594.2025.2569627>
- Lyte, J.M., Seyoum, M.M., Ayala, D., Kers, J.G., Caputi, V., Johnson, T., Zhang, L., Rehberger, J., Zhang, G., Dridi, S., Hale, B., De Oliveira, J.E., Grum, D., Smith, A.H., Kogut, M., Ricke, S.C., Ballou, A., Potter, B., Proszkowiec-Weglarz, M., 2025. Do we need a standardized 16S rRNA gene amplicon sequencing analysis protocol for poultry microbiota research? *Poult. Sci.* 104, 105242. <https://doi.org/10.1016/j.psj.2025.105242>
- Maaz, T.M., Dobermann, A., Lyons, S.E., Thomson, A.M., 2025. Review of research and innovation on novel fertilizers for crop nutrition. *npj Sustain. Agric.* 3, 25. <https://doi.org/10.1038/s44264-025-00066-0>
- Mangotra, A., and Singh, S.K., 2024. Volatile organic compounds: A threat to the environment and health hazards to living organisms – A review. *J. Biotechnol.* 382, 51–69. <https://doi.org/10.1016/j.jbiotec.2023.12.013>
- Masteling, R., de Boer, W., Raaijmakers, J.M., Garbeva, P., Dini-Andreote, F., 2022. Microbial volatiles as mediators of eco-evolutionary dynamics. *Front. Ecol. Evol.* 10. <https://doi.org/10.3389/fevo.2022.960198>
- Metzker, M.L., 2010. Sequencing technologies — the next generation. *Nat. Rev. Genet.* 11, 31–46. <https://doi.org/10.1038/nrg2626>

- Mirzabaev, A., Bezner Kerr, R., Hasegawa, T., Pradhan, P., Wreford, A., Cristina Tirado von der Pahlen, M., Gurney-Smith, H., 2023. Severe climate change risks to food security and nutrition. *Clim. Risk Manag.* 39, 100473. <https://doi.org/10.1016/j.crm.2022.100473>
- Montejano-Ramírez, V., Ávila-Oviedo, J.L., Campos-Mendoza, F.J., Valencia-Cantero, E., 2024. Microbial volatile organic compounds: Insights into plant defense. *Plants* 13, 2013. <https://doi.org/10.3390/plants13152013>
- Morath, S.U., Hung, R., Bennett, J.W., 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biol. Rev.* 26, 73–83. <https://doi.org/10.1016/j.fbr.2012.07.001>
- Muheki, D., Deijns, A.A.J., Bevacqua, E., Messori, G., Zscheischler, J., Thiery, W., 2024. The perfect storm? Co-occurring climate extremes in East Africa. *Earth Syst. Dyn.* 15, 429–466. <https://doi.org/10.5194/esd-15-429-2024>
- Nelson, G., Rosegrant, M., Koo, J., Robertson, R., Sulser, T., Zhu, T., Ringler, C., Msangi, S., Palazzo, A., Batka, M., Magalhães, M., Santos, R.A., Ewing, M., Lee, D., 2009. Climate change: Impact on agriculture and costs of adaptation, food policy. <https://doi.org/10.2499/0896295354>
- Ngalimat, M.S., Mohd Hata, E., Zulperi, D., Ismail, S.I., Ismail, M.R., Mohd Zainudin, N.A.I., Saidi, N.B., Yusof, M.T., 2021. Plant growth-promoting bacteria as an emerging tool to manage bacterial rice pathogens. *Microorganisms* 9, 682. <https://doi.org/10.3390/microorganisms9040682>
- Nie, W., He, Q., Guo, H., Zhang, W., Ma, L., Li, J., Wen, D., 2024. Arbuscular mycorrhizal fungi: Boosting crop resilience to environmental stresses. *Microorganisms* 12, 2448. <https://doi.org/10.3390/microorganisms12122448>
- Nilsson, R.H., Larsson, K.-H., Taylor, A.F.S., Bengtsson-Palme, J., Jeppesen, T.S., Schigel, D., Kennedy, P., Picard, K., Glöckner, F.O., Tedersoo, L., Saar, I., Kõljalg, U., Abarenkov, K., 2019. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res.* 47, 259–264. <https://doi.org/10.1093/nar/gky1022>
- Nunes, M.R., Veum, K.S., Parker, P.A., Holan, S.H., Karlen, D.L., Amsili, J.P., van Es, H.M., Wills, S.A., Seybold, C.A., Moorman, T.B., 2021. The soil health assessment protocol and evaluation applied to soil organic carbon. *Soil Sci. Soc. Am. J.* 85, 1196–1213. <https://doi.org/10.1002/saj2.20244>
- Okoronkwo, D.J., Ozioko, R.I., Ugwoke, R.U., Nwagbo, U.V., Nwobodo, C., Ugwu, C.H., Okoro, G.G., Mbah, E.C., 2024. Climate smart agriculture? Adaptation strategies of traditional agriculture to climate change in sub-Saharan Africa. *Front. Clim.* 6. <https://doi.org/10.3389/fclim.2024.1272320>
- Opik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J.M., Reier, U., Zobel, M., 2010. The online database MaarjAM reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytol.* 188, 223–241. <https://doi.org/10.1111/j.1469-8137.2010.03334.x>
- Oufensou, S., Ul-Hassan, Z., Badr, K., Balmas, V., Jaoua, S., Migheli, Q., 2025. Guns 'N Roses: fungal volatile warfare in postharvest disease control. *Annu. Rev. Phytopathol.* 63, 477–99. <https://doi.org/10.1146/annurev-phyto-120624-122024>
- Owen, G., 2020. What makes climate change adaptation effective? A systematic review of the literature. *Glob. Environ. Change* 62, 102071. <https://doi.org/10.1016/j.gloenvcha.2020.102071>
- Owen, S.M., Clark, S., Pompe, M., Semple, K.T., 2007. Biogenic volatile organic compounds as potential carbon sources for microbial communities in soil from the rhizosphere of *Populus tremula*. *FEMS Microbiol. Lett.* 268, 34–39. <https://doi.org/10.1111/j.1574-6968.2006.00602.x>
- Park, C., Kim, Seung, Choi, S., Kim, Seil, 2021. Comparison of 16S rRNA gene based microbial profiling using five next-generation sequencers and various primers. *Front. Microbiol.* 12, 715500. <https://doi.org/10.3389/fmicb.2021.715500>
- Pascoal, F., Duarte, P., Assmy, P., Costa, R., Magalhães, C., 2024. Full-length 16S rRNA gene sequencing combined with adequate database selection improves the description of Arctic marine prokaryotic communities. *Ann. Microbiol.* 74, 29. <https://doi.org/10.1186/s13213-024-01767-6>
- Peralta Ogorek, L.L., Gao, Y., Farrar, E., Pandey, B.K., 2025. Soil compaction sensing mechanisms and root responses. *Trends Plant Sci.*, Special issue: Root biology and soil health for a sustainable future 30, 565–575. <https://doi.org/10.1016/j.tplants.2024.10.014>

- Perotto, S., and Balestrini, R., 2024. At the core of the endomycorrhizal symbioses: intracellular fungal structures in orchid and arbuscular mycorrhiza. *New Phytol.* 242, 1408–1416. <https://doi.org/10.1111/nph.19338>
- Pettersson, M., and Bååth, E., 2013. Importance of inoculum properties on the structure and growth of bacterial communities during recolonisation of humus soil with different pH. *Microb. Ecol.* 66, 416–426. <https://doi.org/10.1007/s00248-013-0208-1>
- Philippot, L., Chenu, C., Kappler, A., Rillig, M.C., Fierer, N., 2024. The interplay between microbial communities and soil properties. *Nat. Rev. Microbiol.* 1–14. <https://doi.org/10.1038/s41579-023-00980-5>
- Poveda, J., 2021. Beneficial effects of microbial volatile organic compounds (MVOCs) in plants. *Appl. Soil Ecol.* 168, 104118. <https://doi.org/10.1016/j.apsoil.2021.104118>
- Priya, A.K., Alagumalai, A., Balaji, D., Song, H., 2023. Bio-based agricultural products: a sustainable alternative to agrochemicals for promoting a circular economy. *RSC Sustainability* 1, 746–762. <https://doi.org/10.1039/D3SU00075C>
- Pulleman, M., Creamer, R., Hamer, U., Helder, J., Pelosi, C., Pérès, G., Rutgers, M., 2012. Soil biodiversity, biological indicators and soil ecosystem services—an overview of European approaches. *Curr. Opin. Environ. Sustain.*, Terrestrial systems 4, 529–538. <https://doi.org/10.1016/j.cosust.2012.10.009>
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>
- Quiring, S.M., 2009. Monitoring drought: An evaluation of meteorological drought indices. *Geogr. Compass.* 3, 64–88. <https://doi.org/10.1111/j.1749-8198.2008.00207.x>
- Rabot, E., Wiesmeier, M., Schlüter, S., Vogel, H.-J., 2018. Soil structure as an indicator of soil functions: A review. *Geoderma* 314, 122–137. <https://doi.org/10.1016/j.geoderma.2017.11.009>
- Rani, A., Rana, A., Dhaka, R.K., Singh, A.P., Chahar, M., Singh, S., Nain, L., Singh, K.P., Minz, D., 2023. Bacterial volatile organic compounds as biopesticides, growth promoters and plant-defense elicitors: Current understanding and future scope. *Biotechnol. Adv.* 63, 108078. <https://doi.org/10.1016/j.biotechadv.2022.108078>
- Ray Biswas, R. and Rahman, A., 2023. Adaptation to climate change: A study on regional climate change adaptation policy and practice framework. *J. Environ. Manage.* 336, 117666. <https://doi.org/10.1016/j.jenvman.2023.117666>
- Reimann, S. and Lewis, A.C., 2007. Anthropogenic VOCs, in: Volatile organic compounds in the atmosphere. John Wiley & Sons, Ltd, pp. 33–81. <https://doi.org/10.1002/9780470988657.ch2>
- Rosling, A., Eshghi Sahraei, S., Kalsoom Khan, F., Desirò, A., Bryson, A.E., Mondo, S.J., Grigoriev, I.V., Bonito, G., Sánchez-García, M., 2024. Evolutionary history of arbuscular mycorrhizal fungi and genomic signatures of obligate symbiosis. *BMC Genomics* 25, 529. <https://doi.org/10.1186/s12864-024-10391-2>
- Rouphael, Y., Franken, P., Schneider, C., Schwarz, D., Giovannetti, M., Agnolucci, M., Pascale, S. D., Bonini, P., Colla, G., 2015. Arbuscular mycorrhizal fungi act as biostimulants in horticultural crops. *Sci. Hortic.* 196, 91–108. <https://doi.org/10.1016/j.scienta.2015.09.002>
- Salehi-Lisar, S.Y. and Bakhshayeshan-Agdam, H., 2020. Agronomic crop responses and tolerance to drought stress, in: Hasanuzzaman, M. (Ed.), Agronomic Crops: Volume 3: Stress Responses and Tolerance. Springer, Singapore, pp. 63–91. https://doi.org/10.1007/978-981-15-0025-1_5
- Sandrini, M., Nerva, L., Sillo, F., Balestrini, R., Chitarra, W., Zampieri, E., 2022. Abiotic stress and belowground microbiome: The potential of omics approaches. *Int. J. Mol. Sci.* 23, 1091. <https://doi.org/10.3390/ijms23031091>
- Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences* 74, 5463–5467. <https://doi.org/10.1073/pnas.74.12.5463>
- Sangwan, S., and Prasanna, R., 2022. Mycorrhizae helper bacteria: Unlocking their potential as bioenhancers of plant–arbuscular mycorrhizal fungal associations. *Microb. Ecol.* 84, 1–10. <https://doi.org/10.1007/s00248-021-01831-7>
- Sato, H., Mizoi, J., Shinozaki, K., Yamaguchi-Shinozaki, K., 2024. Complex plant responses to drought and heat stress under climate change. *Plant J.* 117, 1873–1892. <https://doi.org/10.1111/tpj.16612>

- Sato, K., Suyama Y., Saito M., and Sugawara K., 2005. A new primer for discrimination of arbuscular mycorrhizal fungi with polymerase chain reaction-denature gradient gel electrophoresis. *GRASSL SCI.* 51, 179–181. doi: 10.1111/j.1744-697X.2005.00023.x
- Sbrana, C., Agnolucci, M., Avio, L., Giovannini, L., Palla, M., Turrini, A., Giovannetti, M., 2022. Mycorrhizal symbionts and associated bacteria: Potent allies to improve plant phosphorus availability and food security. *Front. Microbiol.* 12. <https://doi.org/10.3389/fmicb.2021.797381>
- Schlöter, M., Nannipieri, P., Sørensen, S.J., van Elsas, J.D., 2018. Microbial indicators for soil quality. *Biol. Fertil. Soils* 54, 1–10. <https://doi.org/10.1007/s00374-017-1248-3>
- Schmidt, R., Etalo, D.W., de Jager, V., Gerards, S., Zweers, H., de Boer, W., Garbeva, P., 2016. Microbial small talk: Volatiles in fungal–bacterial interactions. *Front. Microbiol.* 6. <https://doi.org/10.3389/fmicb.2015.01495>
- Schulz-Bohm, K., Martín-Sánchez, L., Garbeva, P., 2017. Microbial Volatiles: Small Molecules with an Important Role in Intra- and Inter-Kingdom Interactions. *Front. Microbiol.* 8. <https://doi.org/10.3389/fmicb.2017.02484>
- Schüßler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycol. Res.* 105, 1413–1421. <https://doi.org/10.1017/S0953756201005196>
- Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. *Genome Biol.* 12, R60. <https://doi.org/10.1186/gb-2011-12-6-r60>
- Semenov, M.V., 2021. Metabarcoding and Metagenomics in soil ecology research: Achievements, challenges, and prospects. *Biol. Bull. Rev* 11, 40–53. <https://doi.org/10.1134/S2079086421010084>
- Shahid, S.A., Zaman, M., Heng, L., 2018. Introduction to Soil Salinity, Sodicity and Diagnostics Techniques, in: Zaman, M., Shahid, S.A., Heng, L. (Eds.), *Guideline for Salinity Assessment, Mitigation and Adaptation Using Nuclear and Related Techniques*. Springer International Publishing, Cham, pp. 1–42. https://doi.org/10.1007/978-3-319-96190-3_1
- Sillo, F., Neri, L., Calvo, A., Zampieri, E., Petruzzelli, G., Ferraris, I., Delledonne, M., Zaldei, A., Gioli, B., Baraldi, R., Balestrini, R., 2024. Correlation between microbial communities and volatile organic compounds in an urban soil provides clues on soil quality towards sustainability of city flowerbeds. *Heliyon* 10. <https://doi.org/10.1016/j.heliyon.2023.e23594>
- Sinore, T., and Wang, F., 2024. Impact of climate change on agriculture and adaptation strategies in Ethiopia: A meta-analysis. *Heliyon* 10, e26103. <https://doi.org/10.1016/j.heliyon.2024.e26103>
- Smith, F.A., Jakobsen, I., Smith, S.E., 2000. Spatial differences in acquisition of soil phosphate between two arbuscular mycorrhizal fungi in symbiosis with *Medicago truncatula*. *New Phytol.* 147, 357–366. <https://doi.org/10.1046/j.1469-8137.2000.00695.x>
- Smith, S.E., and Read, D.J., 2010. *Mycorrhizal Symbiosis*. Academic Press.
- Soil Science Division Staff. 2017. *Soil survey manual*. C. Ditzler, K.Scheffe, and H.C. Monger (eds.). USDA Handbook 18. Government Printing Office, Washington, D.C.
- Sousa, J.A. de J., and Olivares, F.L., 2016. Plant growth promotion by Streptomycetes: ecophysiology, mechanisms and applications. *Chem. biol. technol. agric.* 3, 24. <https://doi.org/10.1186/s40538-016-0073-5>
- Spatafora, J.W., Aime, M.C., Grigoriev, I.V., Martin, F., Stajich, J.E., Blackwell, M., 2017. The fungal tree of life: from molecular systematics to genome-scale phylogenies. *Microbiol. Spectr.* 5, 10.1128/microbiolspec.funk-0053–2016. <https://doi.org/10.1128/microbiolspec.funk-0053-2016>
- Spatafora, J.W., Chang, Y., Gerald L.B., Lazarus, K., Smith, M.E., Berbee, M.L., Bonito, G., Corradi, N., Grigoriev, I., Gryganskyi, A., James, T.Y., O'Donnell, K., Roberson, R.W., Taylor, T.N., Uehling, J., Vilgalys, R., White, M.M., Stajich, J.E., 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108, 1028–1046. <https://doi.org/10.3852/16-042>
- Stockinger, H., Krüger, M., Schüßler, A., 2010. DNA barcoding of arbuscular mycorrhizal fungi. *New Phytol.* 187, 461–474. <https://doi.org/10.1111/j.1469-8137.2010.03262.x>

- Sumbul, A., Ansari, R.A., Rizvi, R., Mahmood, I., 2020. *Azotobacter*: A potential bio-fertilizer for soil and plant health management. *Saudi J. Biol. Sci.* 27, 3634–3640. <https://doi.org/10.1016/j.sjbs.2020.08.004>
- Sun, Y., Robert, C.A., Thakur, M.P., 2024. Drought intensity and duration effects on morphological root traits vary across trait type and plant functional groups: a meta-analysis. *BMC Ecol. Evol.* 24, 92. <https://doi.org/10.1186/s12862-024-02275-6>
- Syed-Ab-Rahman, S.F., Carvalhais, L.C., Chua, E.T., Chung, F.Y., Moyle, P.M., Eltanahy, E.G., Schenk, P.M., 2019. Soil bacterial diffusible and volatile organic compounds inhibit *Phytophthora capsici* and promote plant growth. *Sci. Total Environ.* 692, 267–280. <https://doi.org/10.1016/j.scitotenv.2019.07.061>
- Tang, J., Schurgers, G., Rinnan, R., 2019. Process Understanding of Soil BVOC Fluxes in Natural Ecosystems: A Review. *Rev. Geophys.* 57, 966–986. <https://doi.org/10.1029/2018RG000634>
- Tanji, K.K., 2002. Salinity in the Soil Environment, in: Läuchli, A., Lüttge, U. (Eds.), *Salinity: Environment - Plants - Molecules*. Springer Netherlands, Dordrecht, pp. 21–51. https://doi.org/10.1007/0-306-48155-3_2
- Taye, M.T., and Dyer, E., 2024. Hydrologic extremes in a changing climate: a review of extremes in East Africa. *Curr. Clim. Change Rep.* 10, 1–11. <https://doi.org/10.1007/s40641-024-00193-9>
- Teklu, A., Simane, B., Bezabih, M., 2022. Effectiveness of Climate-Smart agriculture innovations in smallholder agriculture system in Ethiopia. *Sustainability* 14, 16143. <https://doi.org/10.3390/su142316143>
- Tenorio Berrio, R., Nelissen, H., Inzé, D., Dubois, M., 2022. Increasing yield on dry fields: molecular pathways with growing potential. *Plant J.* 109, 323–341. <https://doi.org/10.1111/tpj.15550>
- Trivedi, P., Leach, J.E., Tringe, S.G., Sa, T., Singh, B.K., 2020. Plant–microbiome interactions: from community assembly to plant health. *Nat. Rev. Microbiol.* 18, 607–621. <https://doi.org/10.1038/s41579-020-0412-1>
- United Nations. United Nations Convention to Combat Desertification in Those Countries Experiencing Serious Drought and/or Desertification, Particularly in Africa. Paris: United Nations, 1994. https://www.unccd.int/sites/default/files/2022-02/UNCCD_Convention_ENG_0.pdf
- Usharani, K.V., Roopashree, K.M., Naik, D., 2019. Role of soil physical, chemical and biological properties for soil health improvement and sustainable agriculture. *J. Pharmacogn. Phytochem.* 8, 1256-1267.
- van der Putten, W.H., Bardgett, R.D., Bever, J.D., Bezemer, T.M., Casper, B.B., Fukami, T., Kardol, P., Klironomos, J.N., Kulmatiski, A., Schweitzer, J.A., Suding, K.N., Van de Voorde, T.F.J., Wardle, D.A., 2013. Plant–soil feedbacks: the past, the present and future challenges. *J. Ecol.* 101, 265–276. <https://doi.org/10.1111/1365-2745.12054>
- van Dijk, E.L., Jaszczyszyn, Y., Naquin, D., Thermes, C., 2018. The Third Revolution in Sequencing Technology. *Trends Genet.* 34, 666–681. <https://doi.org/10.1016/j.tig.2018.05.008>
- Vance, C.P., Uhde-Stone, C., Allan, D.L., 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytol.* 157, 423–447. <https://doi.org/10.1046/j.1469-8137.2003.00695.x>
- Verstraete, M.M., Scholes, R.J., Smith, M.S., 2009. Climate and desertification: looking at an old problem through new lenses. *Front. Ecol. Environ.* 7, 421–428. <https://doi.org/10.1890/080119>
- Voroney, R.P., 2007. 2 - THE SOIL HABITAT, in: Paul, E.A. (Ed.), *Soil Microbiology, Ecology and Biochemistry* (Third Edition). Academic Press, San Diego, pp. 25–49. <https://doi.org/10.1016/B978-0-08-047514-1.50006-8>
- Wada, Y., Van Lanen, H. a. J., 2015. Global hydrological droughts in the 21st century under a changing hydrological regime. *Earth Syst. Dyn.* 6, 1–15. <https://doi.org/10.5194/esd-6-1-2015>
- Wang, Q., Liu, M., Wang, Z., Li, J., Liu, K., Huang, D., 2024. The role of arbuscular mycorrhizal symbiosis in plant abiotic stress. *Front. Microbiol.* 14. <https://doi.org/10.3389/fmicb.2023.1323881>
- Wang, X., Chi, Y., Song, S., 2024. Important soil microbiota's effects on plants and soils: a comprehensive 30-year systematic literature review. *Front. Microbiol.* 15. <https://doi.org/10.3389/fmicb.2024.1347745>
- Weng, W., Yan, J., Zhou, M., Yao, X., Gao, A., Ma, C., Cheng, J., Ruan, J., 2022. Roles of Arbuscular mycorrhizal fungi as a biocontrol agent in the control of plant diseases. *Microorganisms* 10, 7. <https://doi.org/10.3390/microorganisms10071266>

- Werner, S., Polle, A., Brinkmann, N., 2016. Belowground communication: impacts of volatile organic compounds (VOCs) from soil fungi on other soil-inhabiting organisms. *Appl. Microbiol. Biotechnol.* 100, 8651–8665. <https://doi.org/10.1007/s00253-016-7792-1>
- White, T., Bruns, T., Lee, S., Taylor, J., Innis, M., Gelfand, D., Sninsky, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: *Pcr Protocols: A Guide to Methods and Applications*, pp. 315–322.
- White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal rna genes for phylogenetics, in: *PCR Protocols*. Elsevier, pp. 315–322. <https://doi.org/10.1016/B978-0-12-372180-8.50042-1>
- Xiao, F., and Zhou, H., 2023. Plant salt response: Perception, signaling, and tolerance. *Front. Plant Sci.* 13. <https://doi.org/10.3389/fpls.2022.1053699>
- Yamada, A., Kahn, L.H., Kaplan, B., Monath, T.P., Woodall, J., Conti, L. (Eds.), 2014. *Confronting Emerging Zoonoses: The One Health Paradigm*. Springer Japan, Tokyo. <https://doi.org/10.1007/978-4-431-55120-1>
- Yang, K., Llusà, J., Preece, C., Tan, Y., Peñuelas, J., 2024. Exchange of volatile organic compounds between the atmosphere and the soil. *Plant Soil* 501, 509–535. <https://doi.org/10.1007/s11104-024-06524-x>
- Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.-H., Whitman, W.B., Euzéby, J., Amann, R., Rosselló-Móra, R., 2014. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat. Rev. Microbiol.* 12, 635–645. <https://doi.org/10.1038/nrmicro3330>
- Zahra, N., Al Hinai, M.S., Hafeez, M.B., Rehman, A., Wahid, A., Siddique, K.H.M., Farooq, M., 2022. Regulation of photosynthesis under salt stress and associated tolerance mechanisms. *Plant Physiol. Biochem.* 178, 55–69. <https://doi.org/10.1016/j.plaphy.2022.03.003>
- Zahran, H.H., 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Mol. Biol. Rev.* 63, 968–989, table of contents. <https://doi.org/10.1128/MMBR.63.4.968-989.1999>
- Zampieri, E., Sillo, F., Metelli, G., Cucu, M.A., Montesano, V., Quagliata, G., Philipp, L., Brescia, F., Conte, A., Giovannini, L., Mennone, C., Fiore, A., Astolfi, S., Savatin, D., Sestili, F., Reitz, T., Balestrini, R., 2025. Insights into the influence of intercropping and arbuscular mycorrhizal inoculation on two modern durum wheat cultivars and their associated microbiota. *Biol. Fertil. Soils* 61, 85–107. <https://doi.org/10.1007/s00374-024-01872-3>
- Zelm, E. van, Zhang, Y., Testerink, C., 2020. Salt tolerance mechanisms of plants. *Annu. Rev. Plant Biol.* 71, 403–433. <https://doi.org/10.1146/annurev-arplant-050718-100005>
- Zhang, M., Zou, Y., Xiao, S., Hou, J., 2023. Environmental DNA metabarcoding serves as a promising method for aquatic species monitoring and management: A review focused on its workflow, applications, challenges and prospects. *Mar. Pollut. Bull.* 194, 115430. <https://doi.org/10.1016/j.marpolbul.2023.115430>
- Zhang, W., Fan, X., Shi, H., Li, J., Zhang, M., Zhao, J., Su, X., 2023. Comprehensive assessment of 16S rRNA gene amplicon sequencing for microbiome profiling across multiple habitats. *Microbiol. Spectr.* 11, e00563-23. <https://doi.org/10.1128/spectrum.00563-23>
- Zhou, G., Zhou, X., Nie, Y., Bai, S.H., Zhou, L., Shao, J., Cheng, W., Wang, J., Hu, F., Fu, Y., 2018. Drought-induced changes in root biomass largely result from altered root morphological traits: Evidence from a synthesis of global field trials. *Plant Cell Environ.* 41, 2589–2599. <https://doi.org/10.1111/pce.13356>
- Zhu, J.-K., 2002. Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53, 247–273. <https://doi.org/10.1146/annurev.arplant.53.091401.143329>

Sitography

Anaconda: <https://anaconda.org/>

One health: <https://www.oneworldonehealth.org/>

Urban Jungle: <https://www.uia-initiative.eu/en/uia-cities/prato>

USDA: <https://www.nrcs.usda.gov/conservation-basics/natural-resource-concerns/soils/soil-health>

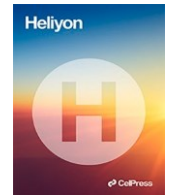
WATDEV: <https://www.watdev.eu/>

Appendix A

In this Appendix the articles in which I contributed as one of the co-authors without the University of Sassari affiliation are attached.

The first work is titled **“Correlation between microbial communities and volatile organic compounds in an urban soil provides clues on soil quality towards sustainability of city flowerbeds”**. The work has been published on Heliyon in 2024.

The second work is titled **“Impact of two *Erwinia* sp. on the response of diverse *Pisum sativum* genotypes under salt stress”**. The work has been published on Physiology and Molecular Biology of Plants in 2024.



Correlation between microbial communities and volatile organic compounds in an urban soil provides clues on soil quality towards sustainability of city flowerbeds

Fabiano Sillo^{a,1}, Luisa Neri^{b,1}, Alice Calvo^a, Elisa Zampieri^a, Gianniantonio Petruzzelli^c, Irene Ferraris^d, Massimo Delledonne^d, Alessandro Zaldei^b, Beniamino Gioli^b, Rita Baraldi^b, Raffaella Balestrini^{a,*}

^a National Research Council, Institute for Sustainable Plant Protection, Strada delle Cacce 73, 10135 Torino, Italy

^b National Research Council, Institute of BioEconomy, Via P. Gobetti 101, 40129 Bologna and Via G. Caproni 8, 50145 Firenze, Italy

^c National Research Council, Institute of Research on Terrestrial Ecosystems (IRET), Via Moruzzi 1, 56124 Pisa, Italy

^d Department of Biotechnology, University of Verona, Strada Le Grazie 15, 37134 Verona, Italy

ARTICLE INFO

Keywords:

Urban environment Bacteria
Fungi
Soil
Soil health
Volatile Organic Compound

ABSTRACT

Soil functionality is critical to the biosphere as it provides ecosystem services relevant for a healthy planet. The soil microbial composition is significantly impacted by anthropogenic activities, including urbanization. In this context, the study of soil microorganisms associated to urban green spaces has started to be crucial toward sustainable city development. Microbes living in the soil produce and degrade volatile organic compounds (VOCs). The VOC profiles may be used to distinguish between soils with various characteristics and management practices, reflecting variations in the activity of soil microbes that use a variety of metabolic pathways. Here, a combined approach based on DNA metabarcoding and GC-MS analysis was used to evaluate the soil quality from urban flowerbeds in Prato (Tuscany, Italy) in terms of microbial biodiversity and VOC emission profiles, with the final aim of evaluating the possible correlation between composition of microbial community and VOC patterns. Results showed that VOCs in the considered soil originated from anthropic and biological activity, and significant correlations between specific microbial taxa and VOCs were detected. Overall, the study demonstrated the feasibility of the use of microbe-VOC correlation as a proxy for soil quality assessment in urban soils.

1. Introduction

In the current Anthropocene era, the importance of soil quality, and its impact on the sustainability of urban environments, is becoming more and more clear as cities continue to develop and expand. As municipalities promote projects implementing green areas intended to restore and enhance ecosystem services, understanding the biological dynamics of urban soil microbiome is becoming a topic of interest not only at scientific level but also for citizen and policy makers [1–3]. The quality of urban soils plays a critical role in

* Corresponding author.

E-mail address: raffaella.balestrini@ipsp.cnr.it (R. Balestrini).

¹ These authors equally contributed as first authors.

<https://doi.org/10.1016/j.heliyon.2023.e23594>

Received 7 July 2023; Received in revised form 6 December 2023; Accepted 7 December 2023

Available online 12 December 2023

2405-8440/© 2023 The Authors.

Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

creating and maintaining urban green spaces, which are essential for promoting environmental sustainability, enhancing human health and well-being, and providing social and economic benefits to urban communities [3]. Urban soils have been defined so far as soils that are heavily mixed with materials that are different from those found in nearby agricultural or forest areas, have a surface layer of more than 50 cm, and have been significantly altered by human activity through mixing, material import and export, and contamination [4]. They are distinguished from rural soils due to the degree of influence of human activities [5]. This classification allows to exclude soils in parks and gardens, since they are regarded as belonging more to agricultural than urban soils [4].

Soils in urban areas are affected by chemical contamination that is recognized as a global threat that involves all environments and is deeply felt in the urban ones, due to the presence of many sources of pollution [6]. For instance, rainwater can dissolve and carry pollutants from the air, including volatile organic compounds (VOCs), and deposit them onto the soil surface [7]. Additionally, road traffic is a source of direct and indirect urban soil contamination because of road dust collection and subsequent atmospheric transfer as well as volatile and particulate emissions from moving cars leading both to deposition into the soil surface and to formation of secondary aerosol [8]. Thus, urban soils are unique and complex systems that are exposed to a multitude of stressors that can have significant impacts on their quality.

Urban soil can be both a sink and source of VOCs [9,10]. Soil VOCs can be divided in two groups: VOCs from anthropic activity origins (AVOCs) and those produced by organisms inhabiting the soil ecosystem, *i.e.*, biogenic volatile organic compounds (BVOCs) [11]. AVOCs, derived from human activities such as combustion, include toxic compounds like BTEX (benzene, toluene, xylene, and related chemicals) and poly-aromatic hydrocarbons (PAHs) [12], and are chemically diverse, encompassing aliphatic and aromatic hydrocarbons, alcohols, alkenes, esters, glycol derivatives, aldehydes, ketones, alkynes and halogenated hydrocarbons [13]. Soil microbes are able to degrade some of these toxic compounds, potentially enhancing plant resistance against contaminants, thanks to their presence [14–17]. BVOCs, on the other hand, are produced by soil organisms, especially plants and microbes [18–20] and include a range of compounds like alcohols, ketones, and terpenes [9,21]. BVOCs can be directly released from soils to the atmosphere through microbial degradation of plant waste or soil organic carbon, and unique BVOC profiles can be associated to distinct microbial taxa [22, 23]. In addition, BVOCs can be used by microorganisms for their metabolism or can be emitted and transported in the environment, acting as signals in plant-plant interaction or plant-microbe interaction [20,24]. Soil-related BVOC emissions also contribute to total BVOC ecosystem emissions and have an impact on atmospheric chemistry [25–27]. However, despite the growing recognition of urban soils as critical components of urban ecosystems, there is a gap in our understanding of the complex interactions between soil microbial communities and synthesis and degradation of VOCs in urban environments. The study of the correlations between microbial communities of urban soil and VOCs has been proposed as pivotal to fill this specific gap [9,28]. This topic is particularly important for several reasons, including assessment of soil quality, sustainable urban development and even air quality in cities. The VOCs produced or degraded by microbes in the soil can provide insights into soil quality and the activity of the soil microbial community [28]. Through the comprehension of the correlation between VOCs and microbial communities, the overall status of the soil, that represents a critical factor for protecting and improving the provided ecosystem services, could be better assessed and evaluated. Since urbanization significantly impacted on soil quality, understanding the relationship between VOCs and microbial communities might help policymakers to take more informed decisions about urban development, promoting sustainable practices and maintaining healthy urban ecosystems. Additionally, soils are a source of VOCs that can contribute to air pollution both directly and through secondary aerosol formation, and affect human health. By understanding the sources and pathways of VOC emissions, strategies to mitigate their impact on the environment could be developed [29].

In this study, whose feasibility was previously demonstrated [28], a combined approach coupling a metabarcoding analysis of microbial communities and VOC emissions in soil was used to evaluate the soil quality of urban flowerbeds in Prato, Italy, in the frame of the project Prato Urban Jungle. The main objective of the project is to create an innovative urban design to re-naturalize certain areas of the Prato municipality in a way that is sustainable and socially inclusive. “Urban jungles”, or regions with a high density of green, are being developed as an approach to boost sustainable urban land use to accomplish this goal. In addition to recover unused soil and space for community use, the urban jungles would improve plants natural capacity to filter pollutants, by converting undesired and decaying locations into green hotspots.

Thus, “Urban jungles” are expected to have net positive impact on soil quality. However, to evaluate the positive effects of the planned activities it is pivotal to evaluate *ex-ante* situation in terms of soil quality, by assessment of soil microbial and VOC diversity. Soils from three different flowerbeds with diverse tree coverage were selected, to assess the role of tree species in shaping soil microbial communities and VOC profiles, and soils from an adjacent park were used to compare data from urban flowerbeds to data from a less disturbed urban area. The overall aim of this work was to identify putative correlation between the composition of the microbial community and the VOC patterns emitted by the selected soils, providing useful information to promote sustainable development in cities.

2. Materials and methods

2.1 Soil sampling

To perform the assessment of the VOCs and the microbial diversity in the selected urban soils, three different sites in the flowerbeds of “Via Turchia”, in a council housing complex of Prato municipality, Tuscany region, Italy, were selected. Each site was selected to have a distinct tree canopy, *i.e.*, *Tilia* spp., *Quercus robur*, and *Quercus ilex*, and were located 75 m far from each other (Fig. S1). Additionally, in an adjacent urban park located 70–130 m from the closest flowerbed, these species were selected in three sites for soil sampling for comparison purpose, *i.e.*, flowerbeds vs park. Sampling was performed in June 2021. For physical-chemical analyses, four

soil samples, for each selected site (flowerbed with *Tilia*, flowerbed with *Quercus robur*, flowerbed with *Quercus ilex*, and urban park) were used. Soil samples of approximately 500 g were collected from the 10 cm layer and air-dried. Soil chemical properties were determined according to standard analytical procedures (Sparks et al., 2020). For metabarcoding and VOC analysis, three points (point 1 close to the tree, point 2 5 m far from the tree, point 3 10 m far from the tree) for each site were selected, and for each point three soil samples (replicates) were collected (A, B, C). Soil samples were collected using a soil Dutch auger. Small debris and litter material were discarded with a hand trowel, and soil cores up to 18 cm deep (around 300 g) were taken. In detail, 27 samples for VOC and metabarcoding analysis from the three different flowerbeds (flowerbed with *Tilia*, flowerbed with *Quercus robur*, flowerbed with *Quercus ilex*) were collected. Additionally, in the urban park, a single sampling corresponding to soil close to each of the three tree species as in flowerbed was selected, for a total of 3 samplings x 3 replicates (9 samples) for metabarcoding and VOC analysis. A total of 36 samples were collected. Sampling point coordinates are reported in Table S1. Soil samples were stored at 4 °C before VOC and metabarcoding analysis. Meteorological data of relative humidity of the sampling day were retrieved from the local weather station of Prato Università. Soil moisture data were retrieved from the Copernicus Global Land Service SSM (Surface Soil Moisture) product, released daily at 1 km spatial resolution and based on the Sentinel-1 C-SAR satellite [30]. The water content of the soil samples collected for the analysis (expressed as percentage) was also measured as $[\text{mass of soil (g)} - \text{mass of oven-dried soil (g)}] / \text{mass of oven-dried soil (g)} \times 100$ [31].

2.2 VOC analysis

2.2.1. Dynamic headspace sampling of volatile compounds

Before VOC analysis, the collected soil samples were homogenized to create a uniform sample [32] and stabilized at ambient temperature [33]. Volatiles were measured using a dynamic headspace (DHS) technique system followed by gas-chromatography–mass spectrometry (GC–MS) analysis based on the procedure developed in our laboratories for different media [28,34], optimized for determination of polar and nonpolar volatile compounds in the headspace of the soil samples and modified as follows. Optimal headspace parameters were: 300 g of soil samples (mainly backfill) transferred into a 500-ml glass container and kept at room temperature for 5 min to reach equilibrium conditions and to prevent alteration of the volatiles; glass container connected via Teflon tubing to a home-made Zero Air Generator, with the gas inlet located at 1 cm from the top of the extraction vessel; outlet of the extraction system connected to steel tubes packed with Tenax TA 35/60 and Carbograph 1 TD 40/60® (Markes International, Ltd, Llantrisant, UK) connected to an external pump (Pocket Pump SKC Inc., USA) adsorbing at flow rate of 200 ml min^{−1} (12 l air collection). To verify the possible presence of VOCs in the ZAG-generated air passing through the entire system, samples of this air flowed into the empty glass container were collected into steel tubes as described above. All the sampled adsorbent tubes were kept at −20 °C until analysis to avoid any chemical alteration and/or artefacts.

2.2.2. Thermal desorption gas chromatography–mass spectrometry (TD-GC–MS) analysis of volatile compounds

GC–MS analysis was carried out as reported in Ref. [35]. In detail, traps were desorbed using a thermal-desorber (Markes International, Series 2 Unity) and analyzed with a 7890A gas chromatograph coupled with a 5975C mass detector (GC–MS, Agilent Technologies, Wilmington, USA). Thermal desorption of the sampling tubes was carried out for 15 min at 280 °C with a helium flow rate of 50 ml min^{−1}, then the cold trap was rapidly heated from −30 °C to 280 °C and analytes were fast injected onto the capillary column (HP-1, 60 m × 0.25 mm I.D. × 0.25 µm film of polymethylsiloxane; J&W Scientific USA, Agilent Technologies, Palo Alto, CA, USA) via a transfer line heated at 280 °C. Identification was obtained by software comparison of retention times and fragmentation pattern with the NIST 11 database of mass spectra and external reference compounds. The identified compounds, corrected by subtracting the VOCs possibly present in the air coming from the ZAG and from the entire sampling system, were quantified using the external standard calibration procedure. Finally, VOC emissions were expressed as ng kg^{−1} h^{−1}.

2.3. Metabarcoding analysis

2.3.1. DNA extraction and sequencing

Genomic DNA from approximately 400 mg of soil from the same sampling points of those collected from VOC analysis was extracted by using DNeasy PowerSoil Pro Kit (Qiagen) following the manufacture procedure. As soil homogenization is a pivotal step of sample preparation to assess microbial communities in soil [36], soil samples were homogenized by using bead-beating tubes provided by the kit and a Vortex Genie® 2 (Scientific Industries) for 20 min. After DNA extraction, the DNA concentration of the 36 samples was measured with a Qubit BR dsDNA assay kit (ThermoFisher). The 16S V3–V4 and the ITS2-5.8S regions were amplified separately and following the 16S Metagenomic Sequencing Library Preparation protocol (Illumina) with 12.5 ng/sample as starting material. Target-specific primers (Table S2) including the Illumina overhang adapter sequence for subsequent sample indexing via a second round of PCR were used. Sample indexing was performed using the Nextera XT Index Kit v2 (Illumina). Final library concentrations were measured with the Qubit HS dsDNA assay kit (ThermoFisher) and used to prepare two equimolar pools (one per each target). The pools were then quantified by Real-Time PCR against a standard curve with the KAPA Library Quantification Kit (Kapa Biosystems). Libraries were sequenced on a MiSeq platform (Illumina) in a 300 paired-end mode. The 16S V3–V4 and the ITS2-5.8S regions were amplified separately for characterization of bacteria and fungi, respectively, following the Metagenomic Sequencing Library Preparation protocol (Illumina). Raw reads were submitted to Sequence Read Archive (SRA) - NCBI (Bioproject ID: PRJNA992400).

2.3.2. Bioinformatic and statistical analysis

The obtained reads were clustered into Amplicon Sequence Variants (ASVs) [37]. Data were analyzed by QIIME2 pipeline and reads from 16S and ITS regions were then classified using the Naïve-Bayes classifiers trained on Silva_v132_99_16S and Unite-v8-99 ITS databases respectively. The analysis on 16S and ITS regions was performed considering all the reads of each sample. The bioinformatic analysis was carried out using the QIIME2_Illumina analysis workflow freely available as a Git repository (https://github.com/swiftbiosciences/q2_ITS). Prior to the analysis, reads were trimmed according to their quality. For the 16S region, the 3' ends of the forward reads were trimmed at base position 269, whereas the reverse reads were trimmed at position 195. Regarding the ITS, forward and reverse reads were trimmed at position 282 and 196, respectively. Output from QIIME2 (feature table and related taxonomy) were used with Microbiome Analyst [38] for visualization and statistical assessment of data. By setting “4” as the minimum quantity of features (10 % of prevalence in samples), as well as 10 % of features with low variance in samples based on IQR, data were filtered to remove low quality and uninformative features. Data rarefying was also carried out to reduce bias due to various library sizes. To assess both fungal and bacterial diversity, Chao1 and Shannon indexes were used to determine the diversity within the samples (α -diversity), while Bray-Curtis was used to compute the diversity between the samples (β -diversity) and Principal Coordinates Analysis (PCoA) was used to show the results. With adjusted p -value cut-off (False Discovery Rate - FDR) set at 0.05 for both analyses (differences were judged significant for p -values of 0.05 or less), analysis of similarities (ANOSIM) was done for β -diversity (at feature level). Functional annotation of bacterial taxa was performed by using FAPROTAX, to associate detected bacterial sequences to their putative metabolic functions [39], while FUNGuild was used to assign trait information based on matching to the classification of fungal taxa [40]. Correlation between VOC, fungal and bacterial taxa was performed by Pearson’s correlation coefficient (r) using R language v 4.2.1 (package “ggpubr”). The database mVOC 3.0 [41] was used to investigate the reported putative association between detected microbial taxa (at genus level) and VOC.

Concerning VOC data, differences in emission rates of VOC categories were assessed by two-way ANOVA without interaction, using as factors the sampling location (the three flowerbeds and the natural park) and the related tree species (*Tilia* spp., *Q. ilex*, *Q. robur*). Tukey post-hoc test was used when ANOVA showed significant differences ($p < 0.05$) among groups. A Principal Component Analysis (PCA) was performed on VOC data to assess the presence of grouped samples and different clusters.

3. Results

3.1. Soil physical-chemical features

The physical-chemical properties of the sampled soils are reported in Table 1. All soil samples could be classified as backfill sandy soils with a low to moderate amount of silt and a low amount of clay, slightly to moderate alkaline pH values and low level of P, and N. Samples from *Tilia* flowerbeds showed and increased amount of N, organic C, and exchangeable Ca and Mg, as well as higher pH (8.04) compared to the other samples (Table 1). The collected urban soil was classified as Calcaric Arenosol [42,43]. During the sampling campaign, the recorded median air relative humidity was 65 % (min 31 % - max 100 %), and the precipitation was 4 mm. Surface Soil Moisture (SSM) reported by Copernicus database was 55 %. Soil water content of the samples was 49.01 ± 8.28 %.

3.2. VOC analysis

Nearly 80 VOCs were identified in the soil samples (Table S3), belonging to different classes. Most abundant chemical classes were acids, alcohols, aldehydes, alkanes, alkenes and arenes, ethers, ketones, terpenes, and terpenoids (Table 2) and further 15 compounds belonging to other different classes (sulphides, furane, pyrrole, phenol, etc.), reported in Table S3. Overall, higher emissions of alcohols, alkanes, alkenes, ketones were detected in samples collected from the park, independently from the tree species, compared to samples from flowerbeds (Fig. 1). Arenes and terpenoids were instead abundant in flowerbed samples (Fig. 1). Acids such as acetic, benzoic and nonanoic, as well as dimethyl disulfide, were high in samples harvested around *Q. robur* in the park (Fig. 1). Terpenes were also particularly detected in samples from the park, specifically in samples close to *Q. ilex*. It should be mentioned that tetrahydrofuran reached high levels in samples coming from soil around *Q. ilex* and *Tilia* in the park (Fig. 1). The PCA on VOC data showed that samples from flowerbed grouped together far from the park samples, except point 2 and 3 of samples of soil close to *Q. robur*. Samples from the park did not cluster together but they were well separated from each other (Fig. 2a). Hierarchical clustering on VOC data, reported in a heatmap (Fig. 2b), showed that samples collected near to *Q. robur*, both in flowerbeds and park, grouped together and separately from those taken close to *Tilia* and *Q. ilex*, except one sample referred to *Tilia* (*Tilia* 1 flowerbed).

Significant differences in VOC emissions were detected by two-way ANOVA. In detail, the amounts of VOCs belonging to class of

Table 1
Physical and chemical features of soil samples. CEC: Cation Exchange Capacity; EC: Electrical Conductivity; org: organic; ex: exchangeable.

Sample	pH	EC	CEC	Clay	Silt	Sand	P	N	C org	Ca ex	Mg ex	Na ex	K ex
		$\mu\text{S}/\text{cm}$	$\text{cmol } ({}^{+})\text{kg}^{-1}$	%	%	%	mg/kg	%	%	g/kg	g/kg	g/kg	g/kg
Park	7.21	505	11.2	9.25	15.5	75.2	12.6	0.12	1.05	1.30	0.19	0.37	0.19
<i>Tilia</i> flowerbed	8.04	560	13.2	6.65	11.3	82.0	13.1	0.21	2.31	2.21	0.21	0.21	0.17
<i>Q. ilex</i> flowerbed	7.73	628	12.8	14.1	9.80	76.1	12.7	0.14	1.26	1.74	0.15	0.35	0.16
<i>Q. robur</i> flowerbed	7.85	425	11.9	12.3	11.4	76.3	11.5	0.11	0.95	1.44	0.10	0.22	0.13

Table 2
Detected VOC classes expressed as average values (ng kg h⁻¹) and related standard deviation. Letters are reported according to outcomes to Tukey HSD test for significant differences (*p*-value < 0.05), if significant differences among group means were detected by ANOVA. The category "OTHERS" includes 15 VOC species different from the other VOC classes.

	<i>Q. ilex</i> park	<i>Q. ilex</i> flowerbed_1	<i>Q. ilex</i> flowerbed_2	<i>Q. ilex</i> flowerbed_3	<i>Q. robur</i> park	<i>Q. robur</i> flowerbed_1	<i>Q. robur</i> flowerbed_2	<i>Q. robur</i> flowerbed_3	<i>Tilia</i> spp. park	<i>Tilia</i> spp. flowerbed_1
Acid	5.8 ± 3.06 a	6.59 ± 5.64 a	9.25 ± 1.53 a	12.78 ± 0.54 a	37.47 ± 17.94 b	9.45 ± 1.14 b	14 ± 5.55 b	17.4 ± 3.42 b	6.5 ± 1.8 a	7.97 ± 6.63 a
Alcohol	9.54 ± 12.57 a	4.7 ± 1.77 b	5.14 ± 2.28 b	3.57 ± 1.14 b	27.44 ± 11.94 a	3.94 ± 2.58 b	11.45 ± 4.95	14.61 ± 11.13 a	32.57 ± 37.8 a	6.73 ± 8.22 b
Aldehyde	6.3 ± 6.21	9.95 ± 0.27	8.11 ± 5.94	6.27 ± 5.25	17.83 ± 8.67	10.8 ± 6.12	17.35 ± 4.59	19.91 ± 9.9	11.03 ± 6.03	10.89 ± 7.92
Alkane	28.33 ± 42.09	22.39 ± 17.01	12.04 ± 16.86	8.71 ± 12.15	33.8 ± 18.12	15.59 ± 20.28	23.62 ± 14.64	25.38 ± 19.26	33.17 ± 50.16	6.6 ± 9
Alkene	4.9 ± 4.2 a	3.17 ± 2.25 a	2.13 ± 1.77 a	1.9 ± 1.5 a	27.66 ± 14.7 b	4.71 ± 3.9 a	6.15 ± 3.48 a	5.54 ± 3.15 a	13.28 ± 4.35 b	2.99 ± 0.03 a
Arene	113.43 ± 181.95	134.66 ± 180.96	66.53 ± 108.93	23.24 ± 30.87	47.45 ± 58.32	371.77 ± 487.26	251.65 ± 228.03	72.2 ± 77.73	69.4 ± 106.26	18.85 ± 17.73
Ether	142.75 ± 224.58	0 ± 0	0 ± 0	0 ± 0	23.14 ± 39.27	0 ± 0	0 ± 0	307.4 ± 361.26	35.2 ± 60.84	23.61 ± 29.1
Ketone	26.84 ± 22.53 a	5.64 ± 6.18 b	6.71 ± 4.8 b	5.43 ± 3 b	15.18 ± 3.51 a	4.03 ± 2.76 b	7.35 ± 5.16 b	8.33 ± 4.53 b	31.79 ± 28.62 a	4.06 ± 3.9 b
Terpene	12.1 ± 18.51	0.64 ± 0.66	0.57 ± 0.57	0.17 ± 0.12	0.22 ± 0.15	0.1 ± 0.12	0.33 ± 0.3	0.26 ± 0.27	1.05 ± 0.57	0.07 ± 0.09
Terpenoid	0.33 ± 0.15 a	0.93 ± 0 b	0.73 ± 0.12 b	0.71 ± 0.09 b	0.26 ± 0.09 a	0.19 ± 0.06 a	0.29 ± 0.09 a	0.33 ± 0.15 a	0.47 ± 0.42 ab	0.74 ± 0.15 b
OTHERS	163.14 ± 159.66	34.19 ± 17.23	27.75 ± 13.24	25.05 ± 7.52	120.99 ± 119.48	33 ± 25.11	394.33 ± 343.37	37.66 ± 21.39	200.15 ± 227.32	13.48 ± 3.45

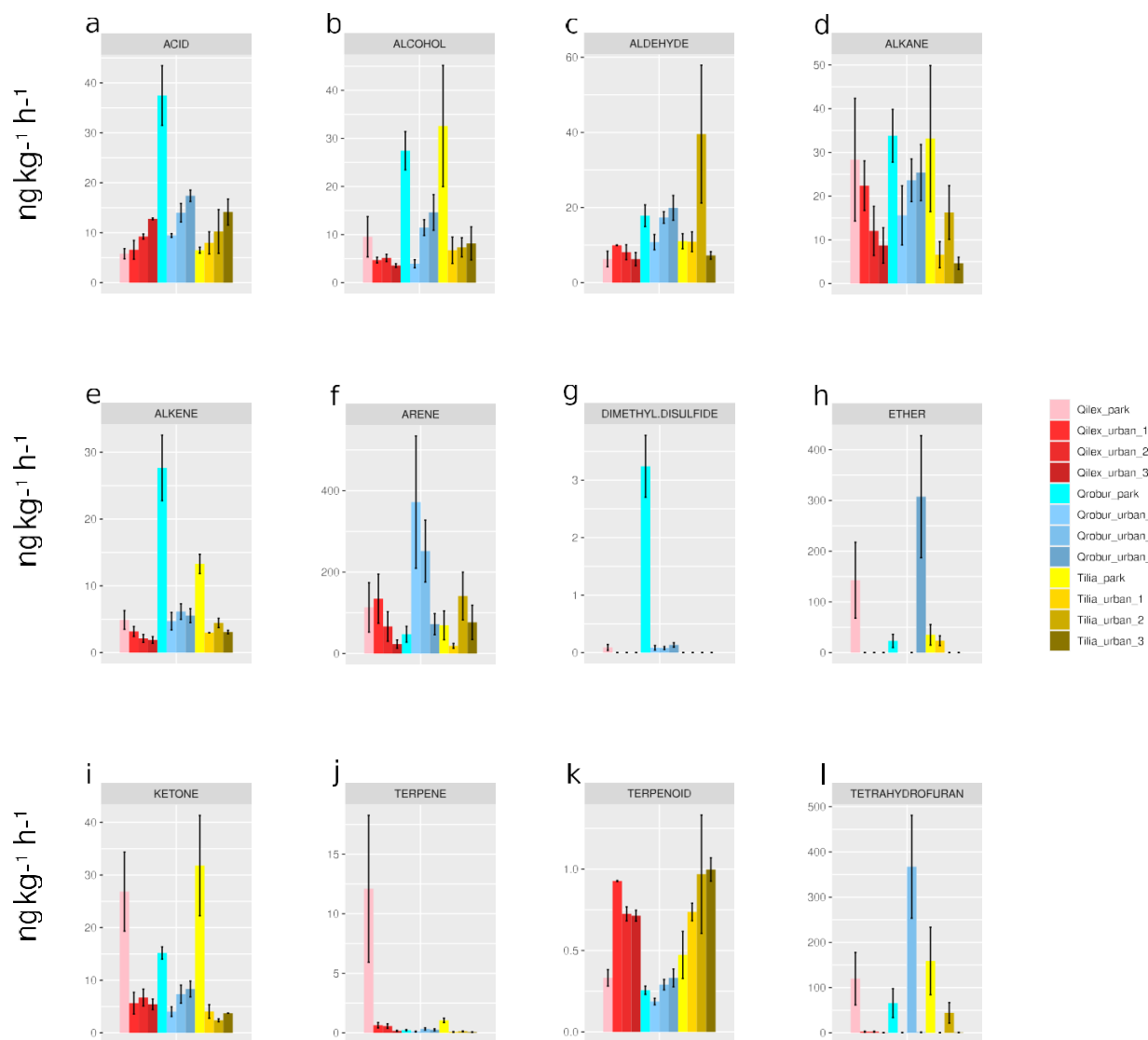


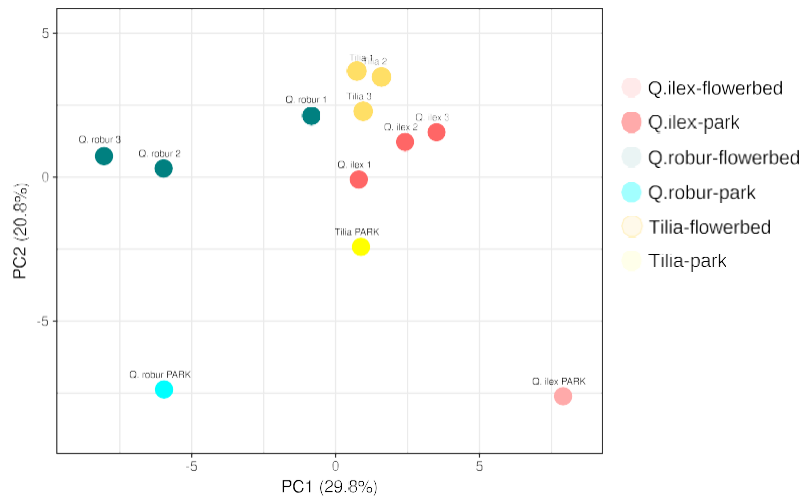
Fig. 1. Barplots of VOC emission from the selected soil samples. VOC species are reported as divided by class with the exception of dimethyl sulfide and tetrahydrofuran. Error bars represent standard errors (SE). Acid (a), alcohol (b), aldehyde (c), alkane (d), alkene (e), arene (f), dimethyl sulfide (g), ether (h), ketone (i), terpene (j), terpenoid (k), and tetrahydrofuran (l) contents are reported. alcohol, alkene and ketone were significantly different between samples collected in flowerbeds and those collected in the urban park (p -value 0.0281, 1.77×10^{-5} , 4.52×10^{-5} , respectively). All these three classes were more abundant in park samples compared to flowerbeds (Fig. 1 and Table 2).

By contrast, acids and terpenoids were different among tree species (p -value 0.00793 and 0.00252, respectively), with higher level of acids and lower level of terpenoids in *Q. robur* samples in comparison with *Tilia* and *Q. ilex* (Fig. 1 and Table 2).

3.3. Microbial community assessment

Metabarcoding of the 16S and ITS genes generated a total of 4,052,368 and 2,772,991 reads after quality filtering, respectively, across the three urban soil sites. Rarefaction curves of both bacterial and fungal ASVs reached a plateau, confirming sufficient sequencing depth for obtaining a whole picture of microbial diversity in the analyzed soil samples. For bacterial data, 1727 ASVs were considered for diversity analysis. The bacterial communities in all samples were dominated by Actinobacteria, Proteobacteria, Acidobacteria, and in less percentage Chloroflexi, Firmicutes, Planctomycetes, Verrucomicrobia and Gemmatimonadetes (Fig. 3a). A relative high presence of Rokubacteria has been observed for some samples collected in the park close to *Quercus ilex* trees (Fig. 3a).

a



b

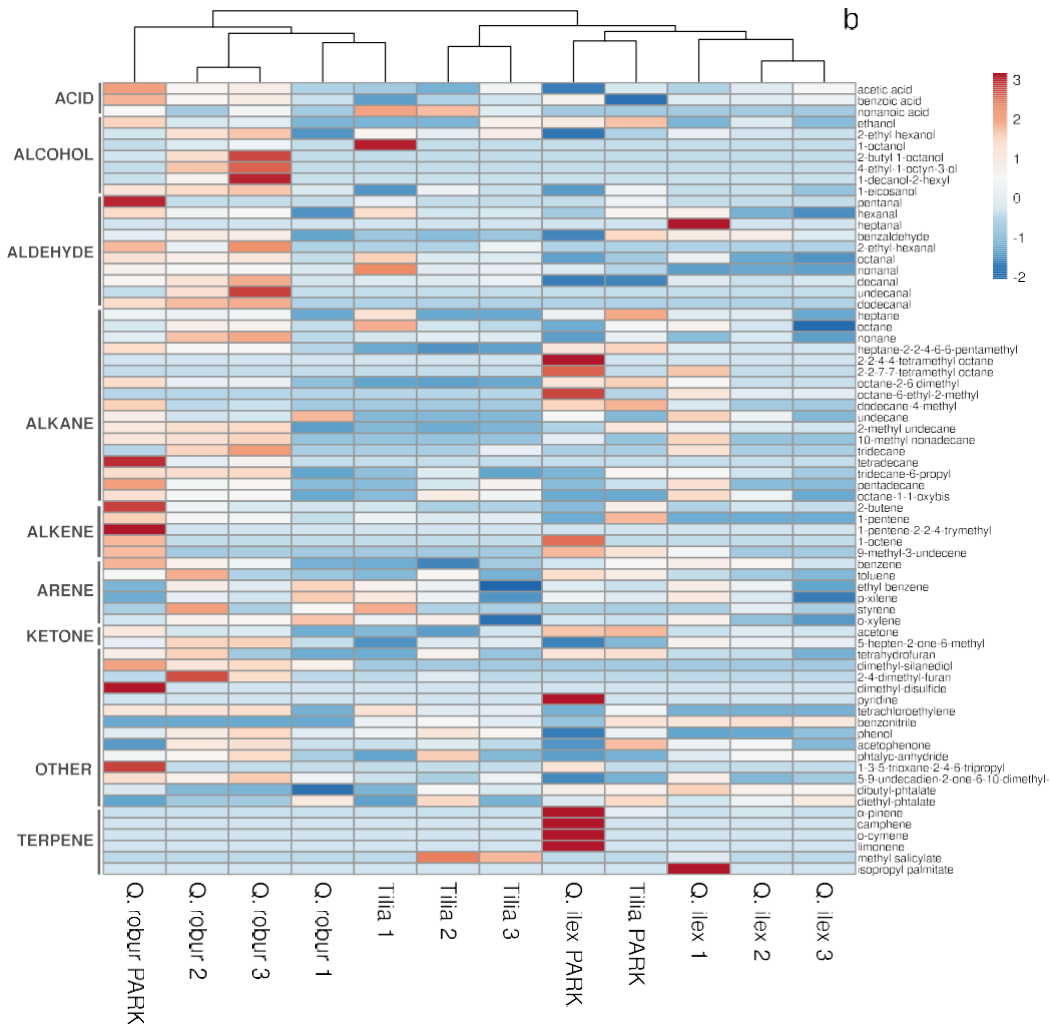


Fig. 2. Total VOC emission profiles. In (a), principal component analysis (PCA) plot of component area normalized data of VOC profiles. In (b), heatmap of the detected VOC species in the selected sampling points. In (b) original values are $\ln(x)$ -transformed, rows are centered and unit variance scaling is applied to rows; columns are clustered using correlation distance and complete linkage.

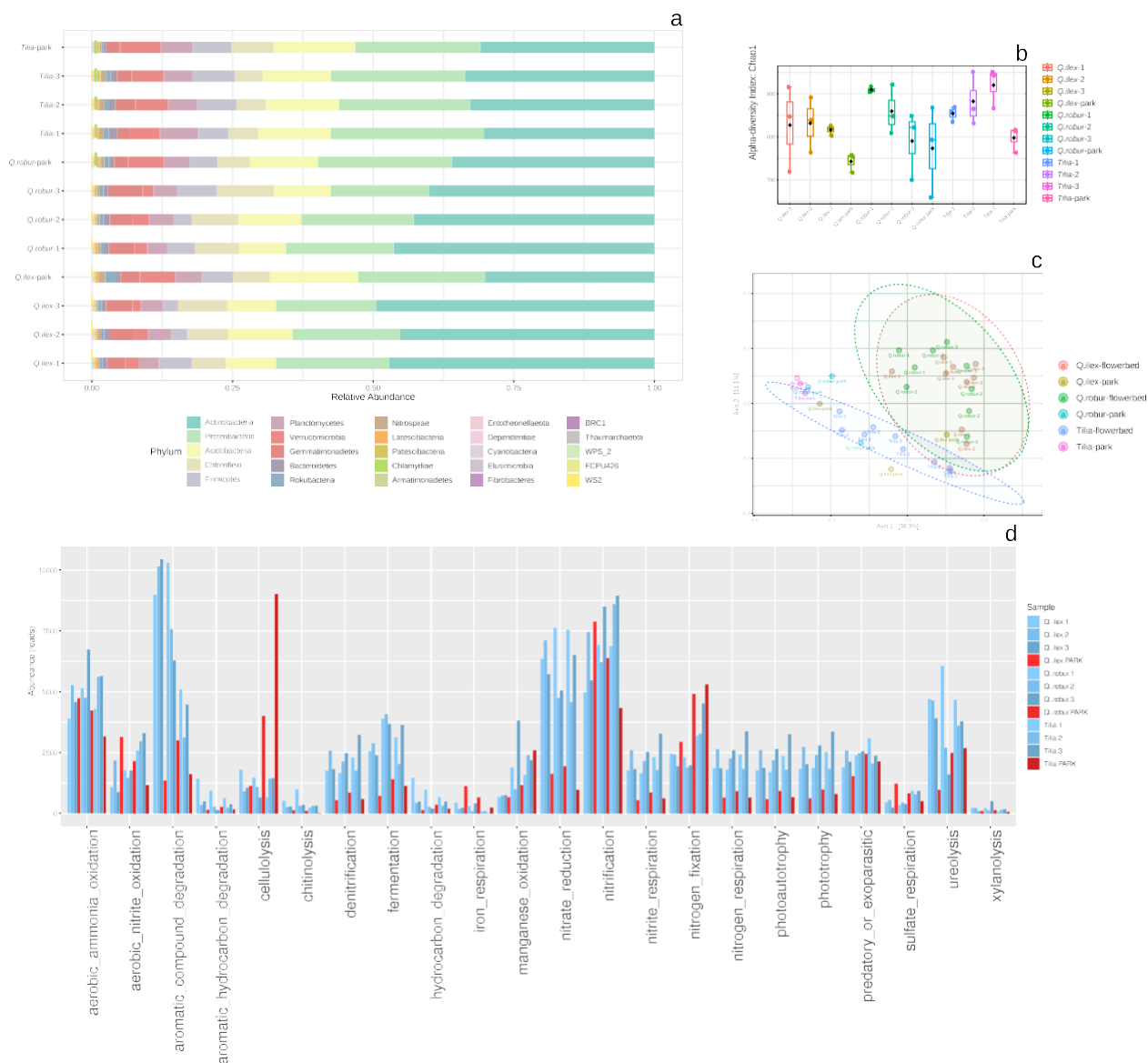


Fig. 3. Results of metabarcoding analysis related to bacteria of soil samples. In (a), stacked taxa barplots of bacterial relative abundance at phylum level in soil samples. In (b) and (c), α - and β -diversity of soil samples calculated with Chao1 diversity index and Bray–Curtis dissimilarity index, respectively. In (d), outcomes of FAPROTAX functional assignment as barplots, where in y-axis is reported the actual abundance and in x-axis the functional classes are listed.

Among Actinobacteria, relevant families were Solirubrobacteraceae (8.47 % of Actinobacteria), Streptomycetaceae (7.80 %), Gaiellaceae (7.40 %), and Nocardioidaceae (7.23 %) (Table S4). Within Proteobacteria, Xanthobacteraceae (21.3 %), Nitrosomonadaceae (6.6 %). Acidobacteria phylum included high percentage of ASVs with not assigned families (98.36 %). Acidobacteria were more abundant in the park samples (T-test FDR 0.019), while Actinobacteria were less detected (T-test FDR 0.018), compared to flowerbed samples.

Alpha-diversity of bacterial communities showed significant differences among samples (Chao1 index, ANOVA F-value 2.230, p -value 0.04). According to this richness index, a higher diversity in flowerbed samples was found when compared to samples from urban park, with the highest α -diversity occurring in samples close to *Tilia* in flowerbed, followed by a sample close to *Q. robur* flowerbed (*Q. robur* 1; Fig. 3b). On the bases on the β -diversity calculated with Bray-Curtis dissimilarity, the bacterial communities in *Q. robur* and *Q. ilex* samples in flowerbeds were more similar to each other than to the bacterial community in *Tilia* samples, and separated from soil samples coming from the park (ANOSIM R 0.57, p -value < 0.001; Fig. 3c). Functional annotation of bacterial communities by FAPROTAX allowed to identify several categories and the most represented are reported in Fig. 3d. High abundance of sequences of bacteria associated with putative “aromatic compound degradation”, “nitrate reduction”, “nitrification”, “aerobic ammonia

oxidation”, and “ureolysis” abilities were detected in flowerbed samples, while samples from the park showed abundance in bacteria putatively capable of “cellulolysis”, “nitrification”, and “nitrogen fixation” (Fig. 3d).

Concerning ITS data, 576 ASVs were defined after filtering, and fungal communities were dominated by Ascomycota, Basidiomycota, and Mortierellomycota (Fig. 4a). Nectriaceae (6.82 % of Ascomycota), Pseudoeurotiaceae (8.26 %) and Aspergillaceae (8.88 %) were the main Ascomycete families; among Basidiomycetes, key families were Cortinariaceae (9.34 % of Basidiomycota), Hymenogastraceae (7.24 %) and Inocybaceae (9.06 %). Mortierellaceae comprised the 100 % of the identified Mortierellomycota fungi (Table S5). No significant differences in α -diversity within samples was observed for fungi, both for Chao1 and Shannon indexes (Fig. 4b). Diversity of fungal communities as determined by β -diversity showed two distinct clusters, one including samples collected in *Quercus* flowerbeds and one with samples collected in *Tilia* flowerbed (Fig. 4c). Samples from the park grouped separately from each other and from the flowerbed samples (Fig. 4c). Guild assignments of trophic modes of detected fungal taxa showed a clear dominance of saprotrophs in all samples, followed by symbiotroph (95 % of Ectomycorrhizal fungi and about 4.2 % of arbuscular mycorrhizal fungi, 324,694 and 14,304 sequences, respectively; Fig. 4d). About 78 % (753,540) of the total sequences (960,884) assigned to Ectomycorrhizal and Arbuscular mycorrhizal fungi were associated to samples close to *Tilia* in flowerbed (Table S6). Comparison by

MicrobiomeAnalyst showed that Basidiomycota and Glomeromycota were significantly more abundant in samples close to *Tilia* tree compared to samples close to *Q. ilex* and *Q. robur* trees (T-test FDR 1.2214×10^{-4} and 3.5633×10^{-3} , respectively).

3.4. Correlation between VOC emissions and microbial communities

Correlation between identified VOCs and microbial communities allowed to detect several bacterial and fungal taxa positively correlated to specific VOCs. Results of correlation analysis at genus level is showed in Fig. 5 and outcomes from correlation between detected microbial species and those reported in mVOC database 3.0 is reported in Table 3, at order level. At genus level, ten bacterial

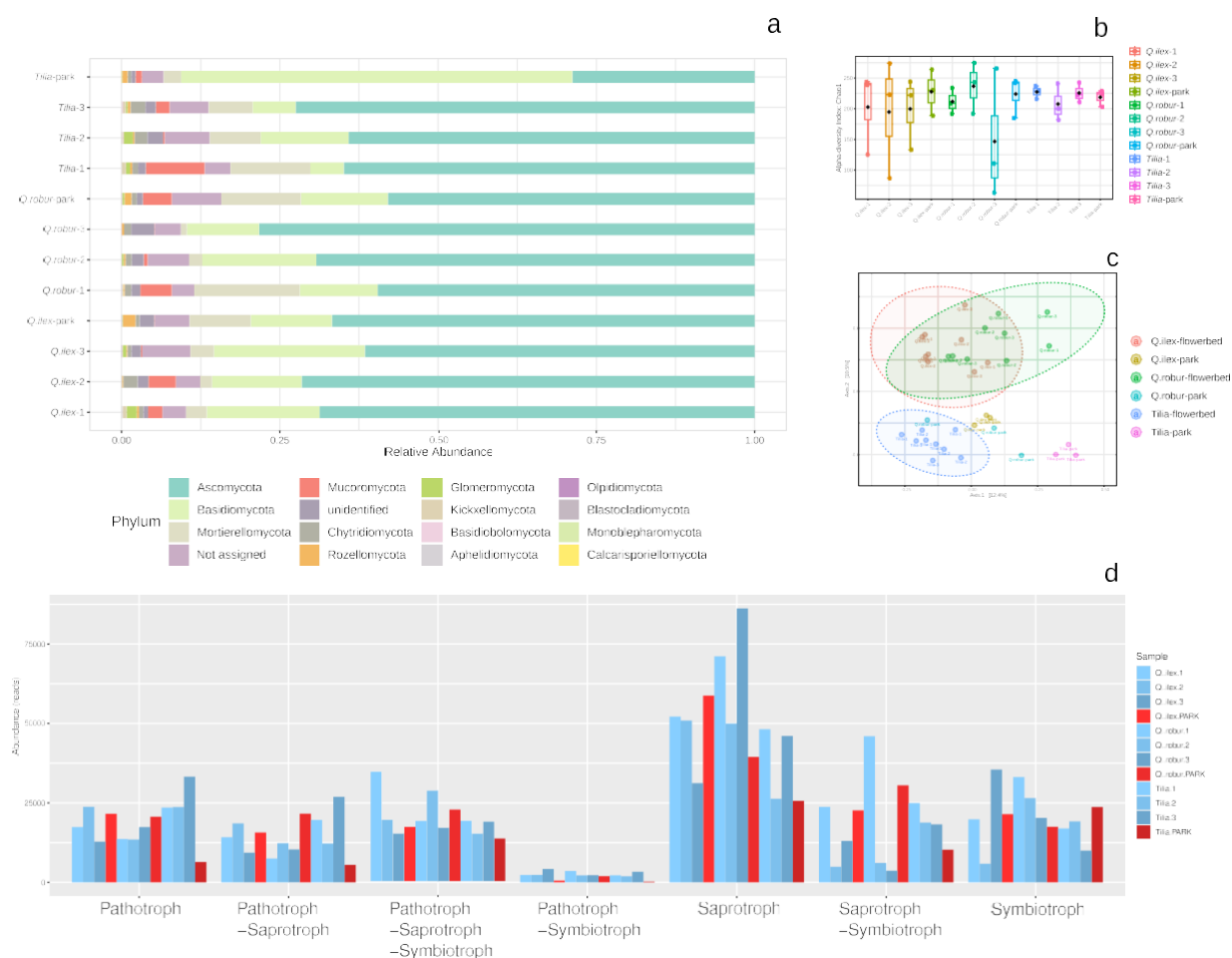
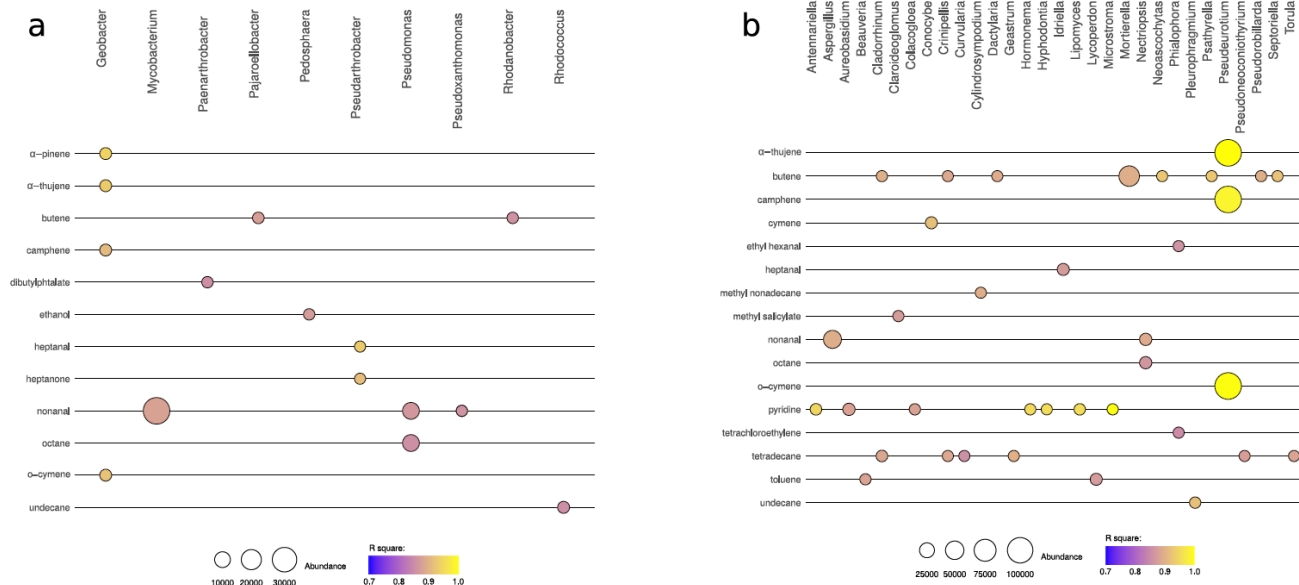


Fig. 4. Results of metabarcoding analysis related to fungi of soil samples. In (a), stacked taxa barplots of fungal relative abundance at phylum level in soil samples. In (b) and (c), α - and β -diversity of soil samples calculated with Chao1 diversity index and Bray–Curtis dissimilarity index, respectively. In (d), results of FUNGuild assignment as barplots, where in y-axis is reported the actual abundance and in x-axis the detected trophic guilds are listed.



taxa were found to be correlated with twelve VOC (Fig. 5a). Among them, *Geobacter*, *Pseudarthrobacter* and *Pseudomonas* correlated with at least two VOC species, with *Geobacter* showing higher R-square values with four VOCs, i.e., α -pinene, α -tujene, camphene and *o*-cymene (Fig. 5a). Nonanal was found to be correlated with three bacterial taxa. Thirty fungal taxa were identified as correlated with sixteen VOC species (Fig. 5b). Fungal clades *Cladorrhinum*, *Crinipellis*, *Nectriopsis*, *Phialophora*, and *Pseudeurotium* were positively associated with at least two VOCs (Fig. 5b). Pyridine, butene and tetradecane showed a correlation with more than five fungal taxa (Fig. 5b).

4. Discussion

4.1. Soil from flowerbed harbored higher microbial diversity compared to the urban park

In recent years, it has been suggested that the restoring and the rewinding of urban green spaces will avoid degradation of microbial communities associated with urban areas, in line with the “Microbiome Rewilding Hypothesis” [44]. However, urban soils may also harbor rich microbial communities, as observed by Scholier and colleagues in a compelling comparative study between urban and natural forests [45]. A similar outcome was observed in our work, where bacterial α -diversity increased in flowerbeds, which can be considered areas with higher level of anthropogenic disturbance compared to the urban park. It has been documented that soil alkalinity plays a role in structuring urban soil microbial communities [45]. In our study site, soil pH of flowerbed samples was slightly higher than in the park. Soil alkalinity has been frequently reported in urban areas [46], and it could be explained by presence of ash emissions and dust from roadways [47,48], or by rainwater flowing through structures as pipes and gutters [49]. In addition, presence of tree species producing base-rich litter, such as linden trees, may have affected the soil pH, as observed in natural forests [50]. For example, in our study, the flowerbed close to *Tilia* trees showed the highest pH compared to the other two flowerbeds. In addition, soil from flowerbed with *Tilia* trees showed a relative high amount of organic carbon and nitrogen, compared to the other flowerbeds where the oak plants were present. Hence, the peculiar microbial communities in soil collected close to *Tilia* trees, as highlighted by beta diversity analysis on both bacterial and fungal ASVs, may be also explained by the high pH and by the abundance of organic matter.

4.2. VOCs in soil from flowerbed originated from anthropic and biological origin

The plethora of detected VOCs from soil collected from the selected flowerbeds suggested the presence of a complex substrate, *i.e.*, urban soils, where belowground processes, such as microbial respiration and exchange of VOCs, occurred [51]. However, and unexpectedly, it has been recently demonstrated that the amounts of various VOCs released from soils rose while the amount of microbial diversity in the soil decreased [52]. In our study, on average, VOC emissions from soil samples of flowerbeds was limited if compared to those from the urban park. Particularly, emission of VOCs belonging to alcohol, alkene and ketone classes was significantly reduced in flowerbeds, despite a higher microbial α -diversity, supporting the observations by Abis and colleagues [52].

By using data from metabarcoding analysis and VOC quantification, significant correlations between several microbial taxa and specific VOCs in the considered soils was inferred. Both fungi and bacteria were found to have a potential role in the generation **Table 3**

Comparison between microbial orders detected in the study and reported in database (mVOC 3.0) as correlated to specific BVOCs.

VOC	Type	Bacterial order in database (mVOC 3.0)	Bacterial order detected as correlated in this study	Fungal order in database (mVOC 3.0)	Fungal order detected as correlated in this study
α -thujene	BVOC	NA	Chthoniobacterales Tistrellales Desulfuromonadales Rokubacteriales	Agaricales Boletales Xylariales	Thelebolales Glomerellales Coniochaetales Sordariales
camphene	BVOC	NA	Tistrellales Desulfuromonadales Chthoniobacterales	Agaricales Boletales Cantharellales Eurotiales Hypocreales Polyporales Tuberales	Thelebolales Coniochaetales Sordariales
cymene	BVOC	NA	Gemmatimonadales	Agaricales Hypocreales Xylariales Tuberales Eurotiales	Agaricales
ethanol	BVOC	NA	Pedosphaerales Blastocatellales	Agaricales Pucciniales Saccharomycetales Tuberales Hypocreales	NA
heptanal	BVOC	NA	Micrococcales	Eurotiales Polyporales Agaricales Hypocreales Tuberales Polyporales	Xylariales Capnodiales
heptanone	BVOC	Streptomycetales	Micrococcales	Polyporales Tuberales	NA
methyl nonadecane	BVOC	Pseudomonadales	NA	NA	Venturiales
methyl salicylate	BVOC	Myxococcales	Xanthomonadales	NA	Glomerales
nonanal	BVOC	NA	Corynebacterales Pseudomonadales Xanthomonadales	Hypocreales Eurotiales Agaricales Tuberales	Eurotiales Hypocreales
<i>o</i> -Cymene	BVOC	Enterobacterales	Chthoniobacterales Tistrellales Desulfuromonadales Rokubacteriales	Eurotiales Hypocreales Polyporales	Thelebolales Glomerellales Coniochaetales Sordariales
pyridine	BVOC	Enterobacterales	NA	Agaricales	Capnodiales Dothideales Dothideales Saccharomycetales Hypocreales Hymenochaetales Microstromatales

degradation of these VOCs [41]. However, both I) the emission of a certain VOC by specific microbial taxa or II) the stimulation of the growth of the microbe when that VOC concentration increases may result in a positive correlation [52]. Our approach based on searching on available VOC database (mVOC3.0) allowed to identify VOC species positively correlated with several fungal taxa, thus suggesting that some detected BVOCs, *i.e.*, α -thujene, heptanal, heptanone, methyl-salicylate, nonanal, pyridine, were putatively emitted by fungi. It is well known that fungi may release complex VOC bouquets, which can affect the growth and overall development of nearby organisms, including plants and bacteria [53]. Abundance of monoterpenoids may be correlated with presence of fungal saprotrophs [23]. Interestingly, in the selected site, large presence of saprotrophs, as highlighted by the FUNGuild assignment, may explain this class of detected BVOCs.

α -thujene is a monoterpene commonly described as produced by plants, especially conifers and *Eucalyptus*, and can be found in many essential oils [54]. The ability of plants to withstand bacterial and fungal disease is also a key function of plant terpenes [55]. However, it has been reported that terpenes are also produced by some fungal species [56,57]. In our study, α -thujene, along with camphene and *o*-cymene, were found to be correlated with the abundance of the ascomycete *Pseudeurotium* spp. This fungal genus has been documented to persist in oil-contaminated environments [58] and to include species able to grow on aromatic compounds [59]. Hence, it is more likely that correlation of this taxa with the emission of several terpenic compounds may result from the ability of these fungi to overcome the putative antifungal activity of these compounds rather than to produce it. Presence of these BVOCs in the soil samples may be linked to emission of terpenic compounds by plant roots [60].

Heptanal is a naturally occurring product of the oxidation of lipids [61]. Due to the presence of yeasts, acetic acid and lactic acid bacteria, this reaction often occurs during fermentation (for example, during the fermentation process for manufacturing bread; [62]). Members of Micrococcaceae were reported to form heptanal as a secondary metabolite [63] and correlation between a member of this family, *i.e.*, *Pseudarthrobacter*, and this VOC may suggest the ability of this genus to produce it. In fungi, heptanal has been reported as produced by *Trichoderma* spp. as antifungal compound against *Neolentinus lepidus*, *Gloeophyllum trabeum*, *Postia placenta* and *Trametes versicolor* [64]. In our study, a link between heptanal and the plant pathogen *Idriella* spp. was discovered. Therefore, it is possible to speculate that this fungus plays a part in the synthesis of this aldehyde, or, alternatively, that heptanal production by bacteria was stimulated by the presence of the fungus. An important BVOC found to be correlated with the presence of mycorrhizal fungal taxa, *i.e.*, *Claroideoglomus* genus, was methyl salicylate. This ester is synthesized by plants *via* the salicylic acid pathway, possibly playing a role in systemic acquired resistance (SAR) under stress conditions [65]. Interestingly, previous studies documented that methyl salicylate emissions increased substantially in plants under high availability of arbuscular mycorrhizal fungi [66]. It could be hypothesized that abundance of the arbuscular mycorrhizal fungus *Claroideoglomus* in urban soils may be linked to methyl salicylate production by nearby plants, or that its presence played a role in the stimulation of its emission. It has been reported that arbuscular mycorrhizal fungi may affect plant VOC emission and that methyl salicylate emission from stressed plants inoculated with arbuscular mycorrhizal fungi increased [67]. An additional BVOC correlating with some bacterial and fungal taxa was nonanal. Nonanal can be produced by a variety of sources, including biological, chemical, and anthropogenic processes [68]. Soil microbes have been shown to produce nonanal through the degradation of organic matter in the soil. Specifically, certain species of bacteria, fungi, and actinomycetes are known to produce nonanal as part of their metabolic processes. In our study, nonanal was correlated with the presence of *Pseudomonas* and *Mycobacterium* genera, and several studies have reported the production of this compound by these bacterial taxa [68–70]. Nonanal is deemed to have an antifungal activity [68]. Lastly, an important significant correlation was detected in our study between the VOC pyridine and several fungal taxa, including both Ascomycetes and Basidiomycetes. Pyridine can be produced by fungi [71], but it could also be a constituent of pollutants as pyridine-based polyurethanes [72]. The observed fungal taxa involved in the generation or degradation of this VOC, regardless of whether it is a real BVOC or derived from anthropogenic origin, should be further investigated.

On the other hand, the presence of bacterial taxa functionally assigned to aromatic compound degradation in flowerbeds may be also indicative of the abundance of potential degraders, suggesting that some AVOCs can be putatively degraded by specific microbial taxa detected in the analyzed soil. Some of the VOCs that have been found and correlated with microbial taxa may indeed have anthropic origins, *i.e.*, dibutyl phthalate, undecane, toluene. Phthalate esters, such as dibutyl phthalate, are typical environmental pollutants and constituents of many polymers [73]. *Microbacterium* spp., *Pandoraea* spp., *Escherichia coli*, *Mycobacterium* spp., *Pseudomonas* spp., and *Sphingomonas* spp. are only a few of the bacteria that may break down phthalate esters [74,75]. One of the most widely used plasticizers is dibutyl phthalate, which is embedded into high-molecular weight polymers like polyvinyl chloride (PVC) [73]. In our study, dibutyl phthalate was found to be highly correlated with abundance of *Paenarthrobacter* genus. It is worth noting that, in contaminated soils, a strain of *Paenarthrobacter* was isolated and characterized as one of the most effective phthalic acid esters degrading bacteria [76], suggesting that the presence of this AVOC in our study site favored this bacterial taxon capable of di-n-butyl phthalate degradation. A similar hypothesis can be inferred for undecane, belonging to the class of alkanes, hydrocarbons with only single bonds between carbon atoms. Undecane is a colorless liquid with a strong odor that is commonly found in petroleum and crude oil, as n-undecane was one of the main VOC constituents in the diesel exhaust [77]. Undecane is considered a VOC because it can easily vaporize and enter the atmosphere, where it can react with other chemicals to form ground-level ozone and contribute to air pollution [77].

4.3. Microbial communities and VOC emissions from soil seem to be associated with the close tree species

The analysis of metabarcoding data highlighted that different microbial communities in urban soils may be correlated with the tree species present in the area, providing useful information for the more suitable species that could be used in the urban green planning. In our study, fungal communities of soil close to linden trees comprised a large presence of symbiotic fungi, including ectomycorrhizal

and arbuscular mycorrhizal fungi. Additionally, soil samples close to *Tilia*, both from park and from flowerbeds, showed a reduced emission of acids and an increased amount of terpenoids. Notably, some terpenoids produced by trees have been demonstrated to have a beneficial impact on human health [78]. This finding raises the question of whether specific plant species recruit certain microbial taxa that can generate or degrade specific VOCs. This hypothesis is supported by studies showing that plants release specific VOCs that can attract or repel specific microbial taxa [79,80]. For example, the species *Thymus vulgaris* releases VOCs that attract specific types of bacteria, which in turn enhance the plant resistance to herbivores [81]. Furthermore, a previous study by Prescott and Grayston [82] showed that the microbial communities in the soil of different tree species were also distinct from each other.

Despite limited on a specific urban area, our findings might have several implications for understanding the relationships between plants and microbial communities in urban environments. First, they suggest that the tree species present in urban areas may have a significant impact on soil microbial communities. This finding is important because microbial communities play a crucial role in maintaining soil health and fertility, and therefore have a direct impact on the health and vitality of urban ecosystems. Second, results suggest that different microbial communities in their associated soil showed the potential to generate or degrade specific types of VOCs. This finding might have important implications for developing sustainable strategies for managing VOC emissions in urban areas, and might boost other research studies in other different urban areas to confirm our outcomes. Although previous studies have suggested that plant roots can be the primary source of VOC emissions from soil [80], our work showed that soil microbes may also play a significant role in this process. The observed emissions were correlated with the abundance and diversity of soil microbial communities independently from the sample origins, thus corroborating the hypothesis that co-occurrence of microbes and VOC species represent a clue of the potential of microbial communities to produce and/or degrade VOCs.

5. Conclusions

In conclusion, our study has shown a significant correlation between microbial communities and specific VOCs in urban soils. The study also demonstrated the feasibility of the use of microbe-VOC correlation as a proxy for soil quality assessment in urban soils. Furthermore, results suggest that the tree species present in urban areas may have a significant impact on the microbial communities in the soil and may have the potential to recruit specific microbial taxa, e.g., the fungi belonging to *Pseudeurotium* spp. and the bacterial taxa *Paenarthrobacter* and *Pseudomonas* spp., that can generate or degrade specific VOC classes.

Data availability statement

Raw reads used in this study were submitted to NCBI Sequence Read Archive (SRA; <https://www.ncbi.nlm.nih.gov/sra>) with Bioproject ID PRJNA992400. All other data presented in the study were included in the supplementary material.

CRediT authorship contribution statement

Fabiano Sillo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Luisa Neri:** Writing – review & editing, Methodology, Investigation, Data curation. **Alice Calvo:** Writing – review & editing, Investigation. **Elisa Zampieri:** Writing – review & editing, Investigation. **Gianniantonio Petruzzelli:** Investigation. **Irene Ferraris:** Investigation, Formal analysis. **Massimo Delledonne:** Investigation, Formal analysis. **Alessandro Zaldei:** Writing – review & editing, Methodology, Conceptualization. **Beniamino Gioli:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Rita Baraldi:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Raffaella Balestrini:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Urban Innovative Actions (UIA) program of the European Commission, under the project PUJ – Prato Urban Jungle, project number UIA04-176. We thank the Municipality of Prato for coordinating the activities and providing access to the sites. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. AC is funded by a grant within WATDEV project (FOOD/2021/425-767).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.heliyon.2023.e23594>.

References

- [1] K. Tzoulas, K. Korpela, S. Venn, V. Yli-Pelkonen, A. Kázmierczak, J. Niemela, P. James, Promoting ecosystem and human health in urban areas using Green Infrastructure: a literature review, *Landsc. Urban Plann.* 81 (2007) 167–178, <https://doi.org/10.1016/j.landurbplan.2007.02.001>.
- [2] A.S. Gill, K. Purnell, M.I. Palmer, J. Stein, K.L. McGuire, Microbial composition and functional diversity differ across urban green infrastructure types, *Front. Microbiol.* 11 (2020) 912, <https://doi.org/10.3389/fmicb.2020.00912>.
- [3] A. Nugent, S.D. Allison, A framework for soil microbial ecology in urban ecosystems, *Ecosphere* 13 (2022), e3968, <https://doi.org/10.1002/ecs2.3968>.
- [4] C.R. De Kimpe, J.L. Morel, Urban soil management: a growing concern, *Soil Sci.* 165 (2000) 31–40, <https://doi.org/10.1097/00010694-200001000-00005>.
- [5] P. Bullock, P.J. Gregory, Soils: a neglected resource in urban areas, in: P. Bullock, P.J. Gregory (Eds.), *Soils in the Urban Environment*, 1991, <https://doi.org/10.1002/9781444310603.ch1>.
- [6] J.L. Yang, G.L. Zhang, Formation, characteristics and eco-environmental implications of urban soils - a review, *Soil Sci. Plant Nutr.* 61 (2015) 30–46, <https://doi.org/10.1080/00380768.2015.1035622>.
- [7] E. Sato, K. Matsumoto, H. Okochi, M. Igawa, Scavenging effect of precipitation on volatile organic compounds in ambient atmosphere, *Bull. Chem. Soc. Jpn.* 79 (2006) 1231–1233, <https://doi.org/10.1246/bcsj.79.1231>.
- [8] R.M. Harrison, J. Allan, D. Carruthers, M.R. Heal, A.C. Lewis, B. Marner, T. Murrells, A. Williams, Non-exhaust vehicle emissions of particulate matter and VOC from road traffic: a review, *Atmos. Environ.* 262 (2021), 118592, <https://doi.org/10.1016/j.atmosenv.2021.118592>.
- [9] J.W. Leff, N. Fierer, Volatile organic compound (VOC) emissions from soil and litter samples, *Soil Biol. Biochem.* 40 (2008) 1629–1636, <https://doi.org/10.1016/j.soilbio.2008.01.018>.
- [10] H. Insam, M.S.A. Seewald, Volatile organic compounds (VOCs) in soils, *Biol. Fertil. Soils* 46 (2010) 199–213, <https://doi.org/10.1007/s00374-010-0442-3>.
- [11] V. Yli-Pelkonen, H. Setälä, V. Viippola, Urban forests near roads do not reduce gaseous air pollutant concentrations but have an impact on particles levels, *Landsc. Urban Plann.* 158 (2017) 39–47, <https://doi.org/10.1016/j.landurbplan.2016.09.014>.
- [12] S.J. Varjani, E. Gnansounou, A. Pandey, Comprehensive review on toxicity of persistent organic pollutants from petroleum refinery waste and their degradation by microorganisms, *Chemosphere* 188 (2017) 280–291, <https://doi.org/10.1016/j.chemosphere.2017.09.005>.
- [13] J. Theloke, R. Friedrich, Compilation of a database on the composition of anthropogenic VOC emissions for atmospheric modeling in Europe, *Atmos. Environ.* 41 (2007) 4148–4160, <https://doi.org/10.1016/j.atmosenv.2006.12.026>.
- [14] A. Smolander, R. Ketola, T. Kotiaho, S. Kanerva, K. Suominen, V. Kitunen, Volatile monoterpenes in soil atmosphere under birch and conifers: effects on soil N transformations, *Soil Biol. Biochem.* 38 (2006) 3436–3442, <https://doi.org/10.1016/j.soilbio.2006.05.019>.
- [15] K.S. Ramirez, C.L. Lauber, N. Fierer, Microbial consumption and production of volatile organic compounds at the soil-litter interface, *Biogeochemistry* 99 (2009) 97–107, <https://doi.org/10.1007/s10533-009-9393-x>.
- [16] N. Das, P. Chandran, Microbial degradation of petroleum hydrocarbon contaminants: an overview, *Biotechnol. Res. Int.*, 2011 (2011) 1–13, <https://doi.org/10.4061/2011/941810>.
- [17] S. Mishra, Z. Lin, S. Pang, W. Zhang, P. Bhatt, S. Chen, Recent advanced technologies for the characterization of xenobiotic-degrading microorganisms and microbial communities, *Front. Bioeng. Biotechnol.* 9 (2021), 632059, <https://doi.org/10.3389/fbioe.2021.632059>.
- [18] T. Zenone, C. Hendriks, F. Brilli, E. Fransen, B. Gioli, M. Portillo-Estrada, M. Schaap, R. Ceulemans, Interaction between isoprene and ozone fluxes in a poplar plantation and its impact on air quality at the European level, *Sci. Rep.* 6 (2016), 32676, <https://doi.org/10.1038/srep32676>.
- [19] J. Tang, G. Schurgers, R. Rinnan, Process understanding of soil BVOC fluxes in natural ecosystems: a review, *Rev. Geophys.* 57 (2019) 966–986, <https://doi.org/10.1029/2018rg000634>.
- [20] M. Simpraga, R.P. Ghimire, D. Van Der Straeten, J.D. Blande, A. Kasurinen, J. Sorvari, T. Holopainen, S. Adriaenssens, J.K. Holopainen, M. Kivimäenpää, Unravelling the functions of biogenic volatiles in boreal and temperate forest ecosystems, *Eur. J. For. Res.* 138 (2019) 763–787, <https://doi.org/10.1007/s10342-019-01213-2>.
- [21] C.M. Gray, R.K. Monson, N. Fierer, Biotic and abiotic controls on biogenic volatile organic compound fluxes from a subalpine forest floor, *J. Geophys. Res. Biogeosci.* 119 (2014) 547–556, <https://doi.org/10.1002/2013jg002575>.
- [22] C.N. Kanchiswamy, M. Malnoy, M.E. Maffei, Chemical diversity of microbial volatiles and their potential for plant growth and productivity, *Front. Plant Sci.* 6 (2015) 151, <https://doi.org/10.3389/fpls.2015.00151>.
- [23] Y. Guo, W. Jud, F. Weigl, A. Ghrardo, R.R. Junker, A. Polle, J.P. Benz, K. Pritsch, J.P. Schnitzler, M. Rosenkranz, Volatile organic compound patterns predict fungal trophic mode and lifestyle, *Commun. Biol.* 4 (2021) 673, <https://doi.org/10.1038/s42003-021-02198-8>.
- [24] M. Saha, P. Gilon, F. Verheggen, Volatile-mediated interactions with surface-associated microbes: a parallelism between phyllosphere of plants and eco- chemosphere of seaweeds, *J. Ecol.* 109 (2021) 2823–2831, <https://doi.org/10.1111/1365-2745.13693>.
- [25] D. Blom, C. Fabbri, E.C. Connor, F.P. Schiestl, D.R. Klauser, T. Boller, L. Eberl, L. Weisskopf, Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions, *Environ. Microbiol.* 13 (2011) 3047–3058, <https://doi.org/10.1111/j.1462-2920.2011.02582.x>.
- [26] T. Mochizuki, Y. Miyazaki, K. Ono, R. Wada, Y. Takahashi, N. Saigusa, K. Kawamura, A. Tani, Emissions of biogenic volatile organic compounds and subsequent formation of secondary organic aerosols in a *Larix kaempferi* forest, *Atmos. Chem. Phys.* 15 (2015) 12029–12041, <https://doi.org/10.5194/acp-15-12029-2015>.
- [27] A.C. Nölscher, A.M. Yañez-Serrano, S. Wolff, A.C. de Araujo, J.V. Lavrić, J. Kesselmeier, J. Williams, Unexpected seasonality in quantity and composition of Amazon rainforest air reactivity, *Nat. Commun.* 7 (2016), 10383, <https://doi.org/10.1038/ncomms10383>.
- [28] L. Neri, F. Sillo, E. Zampieri, E. Lumini, G. Marturano, C.D. Esposti, G. Petruzzelli, B. Gioli, A. Zaldei, R. Baraldi, R. Balestrini, A combined analysis based on microbial communities and volatile organic compounds as a tool to study soil quality in an urban environment, *Pedosphere* 33 (2023) 670–675, <https://doi.org/10.1016/j.pedosph.2023.01.013>.
- [29] Y. Ren, Z. Qu, Y. Du, R. Xu, D. Ma, G. Yang, Y. Shi, X. Fan, A. Tani, P. Guo, Y. Ge, J. Chang, Air quality and health effects of biogenic volatile organic compounds emissions from urban green spaces and the mitigation strategies, *Environ. Pollut.* 230 (2017) 849–861, <https://doi.org/10.1016/j.envpol.2017.06.049>.
- [30] B. Bauer-Marschallinger, V. Freeman, S. Cao, C. Paulik, S. Schauffe, T. Stachl, et al., Toward global soil moisture monitoring with Sentinel-1: harnessing assets and overcoming obstacles, *IEEE Trans. Geosci. Rem. Sens.* 57 (2019) 520–539, <https://doi.org/10.1109/TGRS.2018.2858004>.
- [31] G.C. Topp, G.W. Parkin, T.P.A. Ferr'e, Soil water content, in: M.R. Carter, E.G. Gregorich (Eds.), *Soil Sampling and Methods of Analysis*, Taylor & Francis, Boca Raton, FL, 2008, pp. 939–961, <https://doi.org/10.1201/9781420005271>.
- [32] Y. Jiao, M. Kramshoj, C.L. Davie-Martin, C.N. Albers, R. Rinnan, Soil uptake of VOCs exceeds production when VOCs are readily available, *Soil Biol. Biochem.* 185 (2023), 109153, <https://doi.org/10.1016/j.soilbio.2023.109153>.
- [33] H. Insam, I.H. Franke-Whittle, M. Goberna, Microbes in aerobic and anaerobic waste treatment, in: H. Insam, I.H. Franke-Whittle, M. Goberna (Eds.), *Microbes at Work from Wastes to Resources*, Springer-Verlag, Heidelberg, Germany, 2010, pp. 1–36.
- [34] L. Morrone, E. Vagnoni, N.D. Virgilio, L. Neri, F. Rapparini, G. Bertazza, P. Duce, A. Rotondi, Chemical profiling of bosana virgin olive oils produced in different areas of sardinia, *J. Am. Oil Chem. Soc.* 97 (2020) 1119–1129, <https://doi.org/10.1002/aocs.12402>.
- [35] R. Baraldi, C. Chieco, L. Neri, O. Facini, F. Rapparini, L. Morrone, A. Rotondi, G. Carriero, An integrated study on air mitigation potential of urban vegetation: from a multi-trait approach to modeling, *Urban For. Urban Green.* 41 (2019) 127–138, <https://doi.org/10.1016/j.ufug.2019.03.020>.
- [36] C.A. Müller, L. de Mattos Pereira, C. Lopes, J. Cares, L.G. dos Anjos Borges, A. Giongo, C. Graeff-Teixeira, A. Loureiro Morassutti, Meiofaunal diversity in the Atlantic Forest soil: a quest for nematodes in a native reserve using eukaryotic metabarcoding analysis, *For. Ecol. Manag.* 453 (2019), 117591, <https://doi.org/10.1016/j.foreco.2019.117591>.
- [37] B.J. Callahan, P.J. McMurdie, S.P. Holmes, Exact sequence variants should replace operational taxonomic units in marker-gene data analysis, *ISME J.* 11 (2017) 2639–2643, <https://doi.org/10.1038/ismej.2017.119>.
- [38] J. Chong, P. Liu, G. Zhou, J. Xia, Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data, *Nat. Protoc.* 15 (2020) 799–821, <https://doi.org/10.1038/s41596-019-0264-1>.

- [39] S. Louca, L.W. Parfrey, M. Doebeli, Decoupling function and taxonomy in the global ocean microbiome, *Science* 353 (2016) 1272–1277, <https://doi.org/10.1126/science.aaf4507>.
- [40] N.H. Nguyen, Z. Song, S.T. Bates, S. Branco, L. Tedersoo, J. Menke, J.S. Schilling, P.G. Kennedy, FUNGuild: an open annotation tool for parsing fungal community datasets by ecological guild, *Fungal Ecol.* 20 (2016) 241–248, <https://doi.org/10.1016/j.funeco.2015.06.006>.
- [41] M.C. Lemfack, B.O. Gohlke, S. Toguem, S. Preissner, B. Piechulla, R. Preissner, mVOC 2.0: a database of microbial volatiles, *Nucleic Acids Res.* 46 (2017) D1261–D1265, <https://doi.org/10.1093/nar/gkx1016>.
- [42] E.A.C. Costantini, R. Barbetti, M. Fantappiè, G. L'Abate, R. Lorenzetti, S. Magini, Pedodiversity, in: E.A.C. Costantini, C. Dazzi (Eds.), *The Soils of Italy*, Springer, Dordrecht, Netherlands, 2013, pp. 105–178, https://doi.org/10.1007/978-94-007-5642-7_6.
- [43] Iuss Working Group WrB, World Reference Base for Soil Resources. International Soil Classification System for Naming Soils and Creating Legends for Soil Maps. 4th Edition, International Union of Soil Sciences (IUSS), Vienna, Austria, 2022, p. 234. https://www.isric.org/sites/default/files/WRB_fourth_edition_2022-12-18.pdf.
- [44] J.G. Mills, P. Weinstein, N.J.C. Gellie, L.S. Weyrich, A.J. Lowe, M.F. Breed, Urban habitat restoration provides a human health benefit through microbiome rewinding: the Microbiome Rewilding Hypothesis, *Restor. Ecol.* 25 (2017) 866–872, <https://doi.org/10.1111/rec.12610>.
- [45] T. Scholier, A. Lavrinienko, I. Brila, E. Tukalenko, R. Hindström, A. Vasylenko, C. Cayol, F. Ecke, N.J. Singh, J.T. Forsman, A. Tolvanen, J. Matala, O. Huitu, E. E.
- [46] R.V. Pouyat, I.D. Yesilonis, M. Dombos, K. Szlavecz, H. Setälä, S. Cilliers, E. Hornung, D.J. Kotze, S. Yarwood, A global comparison of surface soil characteristics across five cities, *Soil Sci.* 180 (2015) 136–145, <https://doi.org/10.1097/ss.0000000000000125>.
- [47] S. Godefroid, N. Koedam, Urban plant species patterns are highly driven by density and function of built-up areas, *Landsc. Ecol.* 22 (2007) 1227–1239, <https://doi.org/10.1007/s10980-007-9102-x>.
- [48] T. Magiera, Z. Strzyszczyk, M. Rachwał, Mapping particulate pollution loads using soil magnetometry in urban forests in the Upper Silesia Industrial Region, Poland, *For. Ecol. Manag.* 248 (2007) 36–42, <https://doi.org/10.1016/j.foreco.2007.02.034>.
- [49] P.J. Davies, I.A. Wright, O.J. Jonasson, S.J. Findlay, Impact of concrete and PVC pipes on urban water chemistry, *Urban Water J.* 7 (2010) 233–241, <https://doi.org/10.1080/1573062x.2010.484502>.
- [50] C. Thoms, A. Gättinger, M. Jacob, F.M. Thomas, G. Gleixner, Direct and indirect effects of tree diversity drive soil microbial diversity in temperate deciduous forest, *Soil Biol. Biochem.* 42 (2010) 1558–1565, <https://doi.org/10.1016/j.soilbio.2010.05.030>.
- [51] M. Meischner, S. Haberstroh, L.E. Daber, J. Kreuzwieser, M.C. Caldeira, J. Schnitzler, C. Werner, Soil VOC emissions of a Mediterranean woodland are sensitive to shrub invasion, *Plant Biol.* 24 (2022) 967–978, <https://doi.org/10.1111/plb.13445>.
- [52] L. Abis, B. Loubet, R. Ciuraru, F. Lafouge, S. Houot, V. Nowak, J. Tripied, S. Dequiedt, P.A. Maron, S. Sadet-Bourgeteau, Reduced microbial diversity induces larger volatile organic compound emissions from soils, *Sci. Rep.* 10 (2020) 6104, <https://doi.org/10.1038/s41598-020-63091-8>.
- [53] S. Werner, A. Polle, N. Brinkmann, Belowground communication: impacts of volatile organic compounds (VOCs) from soil fungi on other soil-inhabiting organisms, *Appl. Microbiol. Biotechnol.* 100 (2016) 8651–8665, <https://doi.org/10.1007/s00253-016-7792-1>.
- [54] C. Geron, R. Rasmussen, R. Arnsts, A. Guenther, A review and synthesis of monoterpene speciation from forests in the United States, *Atmos. Environ.* 34 (2000) 1761–1781, [https://doi.org/10.1016/s1352-2310\(99\)00364-7](https://doi.org/10.1016/s1352-2310(99)00364-7).
- [55] J. Gershenzon, N. Dudareva, The function of terpene natural products in the natural world, *Nat. Chem. Biol.* 3 (2007) 408–414, <https://doi.org/10.1038/nchembio.2007.5>.
- [56] S. Breheret, T. Talou, S. Rapior, J.M. Bessière, Monoterpenes in the aromas of fresh wild mushrooms (Basidiomycetes), *J. Agric. Food Chem.* 45 (1997) 831–836, <https://doi.org/10.1021/jf960417h>.
- [57] Ulloa-Benitez, Y.M. Medina-Romero, R.E. Sánchez-Fernández, P. Lappe-Oliveras, G. Roque-Flores, G. Duarte Lisci, T. Herrera Suárez, M.L. Macías-Rubalcava, Phytotoxic and antimicrobial activity of volatile and semi-volatile organic compounds from the endophyte *Hypoxyylon anthochroum* strain Blaci isolated from *Burseria lancifolia* (Burseraaceae), *J. Appl. Microbiol.* 121 (2016) 380–400, <https://doi.org/10.1111/jam.13174>.
- [58] M. Chotard, M.E. Lucchesi, L. Hamouche, S. Tréguer, F. Lelechat, S.L. Floch, J. Mounier, Fungal diversity and surfactant-producing fungi in oil contaminated environments, *J. Appl. Microbiol.* 134 (2022) 1xao070, <https://doi.org/10.1093/jambio/1xao070>.
- [59] F.X. Prenafeta-Boldú, A. Kuhn, D.M. Luykx, H. Anke, J.W. van Groenestijn, J.A. de Bont, Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source, *Mycol. Res.* 105 (2001) 477–484, <https://doi.org/10.1017/s0953756201003719>.
- [60] D. Tholl, Terpene synthases and the regulation, diversity and biological roles of terpene metabolism, *Curr. Opin. Plant Biol.* 9 (2006) 297–304, <https://doi.org/10.1016/j.pbi.2006.03.014>.
- [61] E. Aslankoobi, B. Herrera-Malaver, M.N. Rezaei, J. Steensels, C.M. Courtin, K.J. Verstrepen, Non-conventional yeast strains increase the aroma complexity of bread, *PLoS One* 11 (2016), e0165126, <https://doi.org/10.1371/journal.pone.0165126>.
- [62] S.F. Li, S.B. Zhang, Y.Y. Lv, H.C. Zhai, Y.S. Hu, J.P. Cai, Heptanal inhibits the growth of *Aspergillus flavus* through disturbance of plasma membrane integrity, mitochondrial function and antioxidant enzyme activity, *Lwt* 154 (2022), 112655, <https://doi.org/10.1016/j.lwt.2021.112655>.
- [63] H. Chung, H.J. Seok, Populations of malodor-forming bacteria and identification of volatile components in triolein-soiled cotton fabric, *Fibers Polym.* 13 (2012) 740–747, <https://doi.org/10.1007/s12221-012-0740-5>.
- [64] R.E. Wheatley, The consequences of volatile organic compound mediated bacterial and fungal interactions, *Antonie Leeuwenhoek* 81 (2002) 357–364, <https://doi.org/10.1023/a:1020592802234>.
- [65] S.W. Park, E. Kaimoyo, D. Kumar, S. Mosher, D.F. Klessig, Methyl salicylate is a critical mobile signal for plant systemic acquired resistance, *Science* 318 (2007) 113–116, <https://doi.org/10.1126/science.1147113>.
- [66] A. Velasquez, M. Valenzuela, M. Carvajal, G. Fiaschi, L. Avio, M. Giovannetti, C. D'Onofrio, M. Seeger, The arbuscular mycorrhizal fungus *Funnelliformis mosseae* induces changes and increases the concentration of volatile organic compounds in *Vitis vinifera* cv. Sangiovese leaf tissue, *Plant Physiol. Biochem.* 155 (2020) 437–443, <https://doi.org/10.1016/j.plaphy.2020.06.048>.
- [67] V. Volpe, W. Chittarra, P. Cascone, M.G. Volpe, P. Bartolini, G. Moneti, G. Pieraccini, C. Di Serio, B. Maserti, E. Guerrieri, R. Balestrini, The association with two different arbuscular mycorrhizal fungi differently affects water stress tolerance in tomato, *Front. Plant Sci.* 9 (2018) 1480, <https://doi.org/10.3389/fpls.2018.01480>.
- [68] W.D. Fernando, R. Ramarathnam, A.S. Krishnamoorthy, S.C. Savchuk, Identification and use of potential bacterial organic antifungal volatiles in biocontrol, *Soil Biol. Biochem.* 37 (2005) 955–964, <https://doi.org/10.1016/j.soilbio.2004.10.021>.
- [69] W. Filipiak, A. Sponring, M.M. Baur, A. Filipiak, C. Ager, H. Wiesenhofer, M. Nagl, J. Troppmair, A. Amann, Molecular analysis of volatile metabolites released specifically by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, *BMC Microbiol.* 12 (2012) 113, <https://doi.org/10.1186/1471-2180-12-113>.
- [70] A. Bergmann, P. Trefz, S. Fischer, K. Klepik, G. Walter, M. Steffens, M. Ziller, J.K. Schubert, P. Reinhold, H. Köhler, W. Miekisch, *In Vivo* volatile organic compound signatures of *Mycobacterium avium* subsp. *paratuberculosis*, *PLoS One* 10 (2015), e0123980, <https://doi.org/10.1371/journal.pone.0123980>.
- [71] N. Watanabe, M. Hattori, E. Yokoyama, S. Isomura, M. Ujita, A. Hara, Entomogenous fungi that produce 2,6-pyridine dicarboxylic acid (dipicolinic acid), *J. Biosci. Bioeng.* 102 (2006) 365–368, <https://doi.org/10.1263/jbb.102.365>.
- [72] S. Oprea, V.O. Potolincea, P. Gradinariu, V. Oprea, Biodegradation of pyridine-based polyether polyurethanes by the *Alternaria tenuissima* fungus, *J. Appl. Polym. Sci.* 135 (2017), 46096, <https://doi.org/10.1002/app.46096>.
- [73] F. Zeng, K. Cui, Z. Xie, L. Wu, M. Liu, G. Sun, Y. Lin, D. Luo, Z. Zeng, Phthalate esters (PAEs): emerging organic contaminants in agricultural soils in peri-urban areas around Guangzhou, China, *Environ. Pollut.* 156 (2008) 425–434, <https://doi.org/10.1016/j.envpol.2008.01.045>.
- [74] Z.G. Wang, Y.L. Hu, W.H. Xu, S. Liu, Y. Hu, Y. Zhang, Impacts of dimethyl phthalate on the bacterial community and functions in black soils, *Front. Microbiol.* 6 (2015) 405, <https://doi.org/10.3389/fmicb.2015.00405>.
- [75] Y. Yan, F. Zhu, C. Zhu, Z. Chen, S. Liu, C. Wang, C. Gu, Dibutyl phthalate release from polyvinyl chloride microplastics: influence of plastic properties and environmental factors, *Water Res.* 204 (2021), 117597, <https://doi.org/10.1016/j.watres.2021.117597>.

- [76] S. Shariati, C. Ebenau-Jehle, A.A. Pourbabaee, H.A. Alikhani, M. Rodriguez-Franco, M. Agne, M. Jacoby, R. Geiger, F. Shariati, M. Boll, Degradation of dibutyl phthalate by *Paenarthrobacter* sp. shss isolated from saravan landfill, hyrcanian forests, Iran, *Biodegradation* 33 (2021) 59–70, <https://doi.org/10.1007/s10532-021-09966-7>.
- [77] M. Wang, S. Li, R. Zhu, R. Zhang, L. Zu, Y. Wang, X. Bao, On-road tailpipe emission characteristics and ozone formation potentials of VOCs from gasoline, diesel and liquefied petroleum gas fueled vehicles, *Atmos. Environ.* 223 (2020), 117294, <https://doi.org/10.1016/j.atmosenv.2020.117294>.
- [78] T. Kim, B. Song, K.S. Cho, I.S. Lee, Therapeutic potential of volatile terpenes and terpenoids from forests for inflammatory diseases, *Int. J. Mol. Sci.* 21 (2020) 2187, <https://doi.org/10.3390/ijms21062187>.
- [79] J. Penuelas, M. Staudt, BVOCs and global change, *Trends Plant Sci.* 15 (2010) 133–144, <https://doi.org/10.1016/j.tplants.2009.12.005>.
- [80] K. Schulz-Bohm, S. Gerards, M. Hundscheid, J. Melenhorst, W. de Boer, P. Garbeva, Calling from distance: attraction of soil bacteria by plant root volatiles, *ISME J.* 12 (2018) 1252–1262, <https://doi.org/10.1038/s41396-017-0035-3>.
- [81] M. Maffei, Sites of synthesis, biochemistry and functional role of plant volatiles, *South Afr. J. Bot.* 76 (2010) 612–631, <https://doi.org/10.1016/j.sajb.2010.03.003>.
- [82] C.E. Prescott, S.J. Grayston, Tree species influence on microbial communities in litter and soil: current knowledge and research needs, *For. Ecol. Manag.* 309 (2013) 19–27, <https://doi.org/10.1016/j.foreco.2013.02.034>.



RESEARCH ARTICLE



Impact of two *Erwinia* sp. on the response of diverse *Pisum sativum* genotypes under salt stress

Houda Ilahi^{1,2} · Elisa Zampieri³ · Cristiana Sbrana⁴ · Francesca Brescia³ · Luca Giovannini³ · Roghayeh Mahmoudi⁵ · Gholamreza Gohari⁵ · Mustapha Missbah El Idrissi⁶ · Mohamed Najib Alfeddy⁷ · Martino Schillaci³ · Lahcen Ouahmane⁸ · Alice Calvo³ · Fabiano Sillo³ · Vasileios Fotopoulos⁵ · Raffaella Balestrini³ · Bacem Mnasri²

Received: 21 July 2023 / Revised: 16 February 2024 / Accepted: 20 February 2024 / Published online: 25 February 2024
© The Author(s) 2024

Abstract

Currently, salinization is impacting more than 50% of arable land, posing a significant challenge to agriculture globally. Salt causes osmotic and ionic stress, determining cell dehydration, ion homeostasis, and metabolic process alteration, thus negatively influencing plant development. A promising sustainable approach to improve plant tolerance to salinity is the use of plant growth-promoting bacteria (PGPB). This work aimed to characterize two bacterial strains, that have been isolated from pea root nodules, initially called PG1 and PG2, and assess their impact on growth, physiological, biochemical, and molecular parameters in three pea genotypes (Merveille de Kelvedon, Lincoln, Meraviglia d'Italia) under salinity. Bacterial strains were molecularly identified, and characterized by in vitro assays to evaluate the plant growth promoting abilities. Both strains were identified as *Erwinia* sp., demonstrating in vitro biosynthesis of IAA, ACC deaminase activity, as well as the capacity to grow in presence of NaCl and PEG. Considering the inoculation of plants, pea biometric parameters were unaffected by the presence of the bacteria, independently by the considered genotype. Conversely, the three pea genotypes differed in the regulation of antioxidant genes coding for catalase (*PsCAT*) and superoxide dismutase (*PsSOD*). The highest proline levels (212.88 $\mu\text{mol g}^{-1}$) were detected in salt-stressed Lincoln plants inoculated with PG1, along with the up-regulation of *PsSOD* and *PsCAT*. Conversely, PG2 inoculation resulted in the lowest proline levels that were observed in Lincoln and Meraviglia d'Italia (35.39 and 23.67 $\mu\text{mol g}^{-1}$, respectively). Overall, this study highlights the potential of these two strains as beneficial plant growth-promoting bacteria in saline environments, showing that their inoculation modulates responses in pea plants, affecting antioxidant gene expression and proline accumulation.

Keywords Abiotic stress · Biochemical markers · Pea · 16S rRNA/DNA · RT-qPCR

✉ Raffaella Balestrini raffaellamaria.balestrini@cnr.it

¹ Faculty of Sciences of Tunis, University Tunis El Manar, 2092 Tunis, Tunisia

² Laboratory of Legumes and Sustainable Agroecosystems, Centre of Biotechnology of Borj-Cédria, BP 901, 2050 Hammam-Lif, Tunisia

³ Institute for Sustainable Plant Protection (IPSP), National Research Council of Italy, Strada Delle Cacce 73, 10135 Turin, Italy

⁴ Institute of Agricultural Biology and Biotechnology (IBBA), National Research Council of Italy, Via Moruzzi 1, 56124 Pisa, Italy

⁵ Department of Agricultural Sciences, Biotechnology and Food Science, Cyprus University of Technology, 3036 Limassol, Cyprus

⁶ Faculty of Sciences, Centre de Biotechnologies Végétale et Microbienne, Biodiversité et Environnement, Mohammed V University in Rabat, Rabat, Morocco

⁷ Phytobacteriology Laboratory Plant Protection Research, Unit CRR Marrakesh National Institute for Agronomical Research Marrakesh, 40000 Marrakesh, Morocco

⁸ Laboratory of Microbial Biotechnologies Agrosiences and Environment, Cadi Ayyad University, 40000 Marrakesh, Morocco

Introduction

Leguminous species such as pea (*Pisum sativum*) are an important source of nutritional components due to the seed richness in proteins, carbohydrates, vitamins, minerals, fibers and antioxidant compounds (Sapre et al. 2022). Pea can be grown in different regions, and it is fourth in legume global production after soybean, peanut, and dry beans (Vidal-Valverde et al. 2003). It is an important food and fodder legume for Mediterranean countries, including Tunisia, due to high nutritional value of their protein and starch-rich seeds (El Idrissi et al. 2020). Being a legume species, pea cultivation increases the content of nitrogenous compounds in nitrogen-poor soils (Kebede 2021). However, throughout the course of the five years from 2017 to 2021, pea output worldwide decreased by around 10% as a result of declining soil productivity and harvested area overall (FAOSTAT 2021). This scenario will be exacerbated by the increase of food demand (from 35 to 56%) globally, and the subsequent need to achieve increased crop yields under the world growing population (van Dijk et al. 2021). Addressing this challenge needs effective agricultural management to both enhance crop resilience to abiotic stresses and to preserve soil fertility and health, especially under environmental constraints (van Dijk et al. 2021).

Arid and semi-arid areas are among the most vulnerable to environmental constraints that are exacerbated by climate change. Salinity is a relevant environmental limiting element, causing major reductions in plant productivity (Peng et al. 2023; Raza et al. 2022). It is estimated that more than 50% of the arable soils in the world will be salinized by 2050 (Etesami et al. 2022). In Tunisia, about 25% of the cultivated area is affected by salinity, and it is evaluated that 9.13% of the total Tunisian surface is challenged by this threat due to aridity and poor water management (Aloui et al. 2022). The growth of crops is negatively affected by salt stress, which causes osmotic stress due to decreased water availability and ion toxicity from nutritional imbalances (Acosta-Motos et al. 2017).

Although *P. sativum* is recognized to be moderately tolerant to soil salinity (Shahid et al. 2022), elevated salt levels in soil were reported to significantly affect its productivity (Noreen and Ashraf 2009). In previous few years, inoculation of plants with beneficial soil microbes, including plant growth-promoting bacteria (PGPB), has proven to be a useful approach to enhance sustainable agriculture in soils subjected to several abiotic stresses, including salinity (Bhat et al. 2023, 2020; Peng et al. 2023; Mishra et al. 2021). They can increment the germination of seeds as well as the leaf size, improve chlorophyll and protein amount, increase crop growth and yield, in addition to

nutrient accessibility, and delay the senescence of leaves (Saghafi et al. 2019). They ameliorate salt stress tolerance by different mechanisms, *i.e.*, synthesizing antioxidant enzymes, non-enzymatic antioxidants, and osmolytes (*e.g.*, proline), improving nutrient uptake, producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole acetic acid (IAA), siderophores and exopolysaccharide (EPS; Peng et al. 2023; Saghafi et al. 2019). Endophytic bacteria, such as *Bacillus subtilis* and *Pseudomonas fluorescens*, significantly decreased Na^+ accumulation and promoted K^+ uptake in pea under salt stress, affecting osmoregulation and antioxidant capacity (Sofy et al. 2021). Plant salt tolerance can be affected by the capacity of microbes to regulate the expression of plant transcription factors involved in stress responses, additionally to the synthesis of enzymes correlated to reactive oxygen species (ROS), proline and the synthesis of EPS (Aeron et al. 2020). It was observed that PGPB may improve plant development in a way that depends on the bacterial strain and plant genotype, as different plant genotypes may differentially react to the same microbial strain (Wei and Jousset 2017). Several reports have shown the potential of PGPB in improving productivity of many legume plants, including peanuts (Sharma et al. 2016), *Sulla carnosa* (Hidri et al. 2016) and *Lathyrus cicera* (Gritli et al. 2022) under salt stress conditions. Inoculation with *Planomicrobium* sp. MSSA-10 significantly increased pea growth under salt stress, decreasing ROS and enhancing antioxidant enzyme activities (Shahid et al. 2018). *Variovorax paradoxus* 5C-2 has been found to mitigate salt stress in pea, enhancing water relations, ion homeostasis and photosynthesis (Wang et al. 2016), while the application of *P. sativum* with *Bacillus marisflavi* (CHR JH 203) and *Bacillus cereus* (BST YS1_42) affected the expression of genes involved in ROS scavenging, defense and cell rescue, and enhanced growth and tolerance to the stress itself in greenhouse under salt stress (Gupta et al. 2021a).

Several bacterial species have been characterized as PGPB, including species belonging to genera known to be plant pathogens (Wei et al. 2023; Passera et al. 2019). Among them, *Pseudomonas syringae* is a bacterial genus that include strains with beneficial effect on plants (*i.e.*, *P. syringae* pv. *syringae* strain 260-02) and others that, on the contrary, are pathogenic ones (*i.e.*, *P. syringae* pv. *tomato* strain DC3000) (Passera et al. 2019). It is known that *Erwinia* species may cause fire blight to Rosaceae plants (*e.g.*, *E. amylovora*), and bacterial blight to cucurbits and other dicotyledonous plants, soft rot in a broad-host-range (*E. carotovora*), but also show positive associations with plants such as tea, rice and wheat (*e.g.*, *E. tasmaniensis* and *E. billingiae*) (Jia et al. 2022; Sagar et al. 2018). The application of *Erwinia* sp. as PGPB significantly improved

tomato fruit fresh and dry weight and yield compared with *Bacillus pumilus* and *P. putida* (Shen et al. 2012).

This study was aimed to verify the impact of two strains of *Erwinia* sp., previously isolated from pea root nodules, on alleviating salt stress in three *P. sativum* genotypes, using both two cultivars largely employed in Tunisia (Merveille de Kelvedon and Lincoln) and one dwarf genotype commercialized in Italy (Meraviglia d'Italia). The effects of the bacterial inoculation on pea response to a salt stress condition were evaluated by considering biometric parameters, biochemical traits, and expression of genes previously correlated with pea salt stress response.

Materials and Methods

Isolation of the bacterial strains

Soil of two fields in Tunisia served as the source of the pea nodule associated bacterial strains. Briefly pea plants (Merveille de Kelvedon cultivar) were grown using soil samples from two northern Tunisian locations (Mograne and Zaghwan) for capturing bacteria found in the native soil (Ilahi et al. 2021). For each region, three soil samples were collected at a distance of 50 m apart from each other and they were mixed to obtain a pooled sample by location. Pea seeds were sterilized and germinated as described by Gritli et al. (2019). The seedlings were grown in a greenhouse for fifty days upon the natural light with a daily temperature of 20–24 °C (minimum–maximum). At the end of the growing period, root nodules were collected and sterilized on the surface according to Vincent's standard procedures (Mnasri et al. 2009). Bacterial strains were isolated from collected root nodules by selecting individual colonies on mannitol yeast agar (YEMA) (Vincent 1970). The isolation of 20 isolates, grown on YEMA-RC (Red Congo) medium at 28 °C, allowed to distinguish two different groups of bacterial strains. The first majority group (16/20) was composed of bacteria with slow growth (appearance of colonies after four days). The second minority group (4/20) included fast-growing bacteria (appearance of colonies after two days). Two strains (called PG1 and PG2), belonging to the second group (*i.e.*, both showing a fast growth), were selected for biochemical and molecular characterizations.

Estimation of PGP activities in vitro

Different biochemical capabilities have been assessed in PG1 and PG2 strains, regarding the inorganic phosphate solubilization, the capacity to produce indolacetic acid (IAA) and ammonia (NH₃), the utilization of L-aminocyclopropane-L-carboxylic acid (ACC), and the possibility

to grow in diverse stressed conditions such as osmotic and saline environment.

To test the capacity of bacteria to solubilize inorganic phosphate, bacterial cell suspension (5 µL, 1×10^8 CFU mL⁻¹) was spotted on plates with Pikovskaya agar medium with tri-calcium phosphate (Ca₃(PO₄)₂), considering a triplicate for each plate. Following an incubation at 28 °C for 72 h, bacterial colonies with the clarification haloes was considered as positive for phosphate solubilization activity, and diameter of haloes were evaluated by Fiji software (ImageJ1.50i; Schneider et al. 2012).

Production of IAA was measured by using a colorimetric detection assay in liquid culture (Karnwal 2009). Isolates were cultured in Luria–Bertani (LB) broth, and then 300 µL of bacteria suspension (adjusted to 1×10^8 CFU mL⁻¹) were inoculated in 15 mL tubes filled with 3 mL of DF (Dworkin and Foster 1958) salt minimal broth with 150 µg mL⁻¹ of L-Tryptophan. Tubes were maintained at 28 ± 2 °C on an orbital shaker (200 rpm, 48 h) in dark conditions. One mL of cell suspensions was centrifuged (4,000 rpm, 20 min, 4 °C) and 250 µL of supernatant were combined with 1 mL of Salkowski's reagent (1.2% FeCl₃ in 37% sulphuric acid). Samples were incubated twenty minutes at room temperature, using 96-well polystyrene dishes. A FluoStar Omega microplate reader was used to measure the absorbance of bacterial suspensions (Brescia et al. 2023). IAA concentrations were determined by the preparation of a standard curve using a range of 0.5–100 µg mL⁻¹ of pure IAA, expressing the IAA amount in µg produced by bacterial suspensions at 0.5 McFarland scale density (1.5×10^8 cells mL⁻¹).

Evaluation of the utilization of ACC by bacterial strains (Li et al. 2011) was performed using 2 mL aliquots of LB cultures that were centrifuged at 8000 g for 5 min. The resulting pellets were then washed two times with 1 mL of DF medium (Dworkin and Foster 1958) and resuspended in 2 mL of DF medium with 3 mM ACC. Cultures were incubated at 28 °C, using an orbital shaker (200 rpm), for 24 h and 1 mL of each culture was then centrifuged at 8000 g for 5 min. One hundred µL of the supernatants (1:10 in DF medium) and 200 µL of each diluted supernatant, in addition to 200 µL of DF + ACC medium that was used as reference, were mixed to 400 µL of ninhydrin reagent and boiled for 30 min. Three aliquots of 180 µL for each strain as well as the reference samples were placed in the 96-well polystyrene plates, and the resulting Ruhemann's Purple colour was evaluated by using a FluoStar Omega microplate reader (570 nm absorbance). A standard curve (in the range 0.005 and 0.05 mM) was used to assess ACC amount. In detail, the ACC-utilizing strains were grouped calculating the % of residual ACC compared with that of the DF-ACC medium without inoculation (Brescia et al. 2023). Production of ammonia (NH₃) by the considered bacterial cultures were evaluated by using Nessler's reagent (Cappuccino

and Sherman 1999), following both the Abdelwahed et al. (2022) modified protocol and the original method. The two considered bacterial strains were grown in triplicate in peptone broth (10 mL) for 48 h in an incubator shaker at 30 ± 0.1 °C. After centrifugation (5 min, 6000 g), aliquots of 100 µL of each bacterial supernatant were mixed with 200 µL of Nessler's reagent in 1.5 mL tubes and the mixture was incubated for 10 min, during which its colour turned to yellow to dark brown. After mixing, 33 µL aliquots of each reaction mixture were transferred to the wells in a 96-well plate and diluted with 198 µL of ultra pure water, and a FluoStar Omega microplate reader was utilized (450 nm absorbance) for estimating the extracellular production of NH_3 . The absorbance of a standard reference curve, prepared using ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) solutions (from 50 to 400 µM), was read along with samples. In the original method, greater amounts of bacterial supernatants (1 mL), Nessler's reagent (9 mL) were utilized, keeping the same reaction timing and dilution ratio before transferring the samples to 1-mL cuvettes for absorbance measurements. The two strains' capacity to grow in osmotic and saline environments were also checked. The osmotic stress conditions were produced by using polyethylene glycol (PEG 6000) (Bandeppa et al. 2015). Bacterial strains were inoculated in liquid nutrient broth with glucose (NBG) medium containing 10%, 20%, 30%, 40% and 50% PEG 6000 (100 µL of a suspension at $\text{OD}_{600} = 1$) and put in a shaking incubator set at 28 °C and 120 rpm. NBG medium without PEG 6000 was used as a control.

To evaluate the growth in salt stress conditions, colonies were streaked on nutrient glucose agar (NGA) plates containing 2.5%, 5%, 7.5% or 10% NaCl (Nautiyal et al. 2000). NGA plates without any addition of NaCl (*i.e.*, 0.5% NaCl) were used as control. Samples were kept at 28 °C (in the dark), and bacterial growth was checked after 72 h. Number of replicates was three ($n = 3$) for each test. Putative pathogenicity of the two bacterial strain was also tested on tomato plants (Supplementary Information file).

Biocontrol activity of bacterial strains against phytopathogens

Selected strains were checked for their capacity to inhibit the growth of *Fusarium oxysporum* f. sp. *lentis* and *Rhizoctonia solani* (provided by CNR-ISPA ITEM collection, Bari, Italy, and Dr. Alessandro Infantino, CREA-DC, Roma, Italy, respectively). Six-mm-diameter mycelial plugs obtained from 6-day-old cultures of the two fungal phytopathogens were put in the middle of a 9-cm-diameter Petri dish containing potato dextrose agar (PDA). After growing the bacterial strains in 2 mL of nutrient broth (NB) on an orbital shaker (200 rpm, 24 h, 28 °C), they (once that they have reached an optical density to $\text{OD}_{600} = 1$) were streaked (10 µL) at the

opposite sides (about 7 cm apart) of PDA plates containing the fungus. Plates containing the sole fungal isolate were used as controls. Each bacterial strain and each fungal control were cultured in triplicate for 6 days at 25 °C. The diameters of fungal colonies were evaluated to calculate the inhibition rate (% of $1 - dt/dc$, where dt is the mean test fungal colony diameter and dc is the control one).

Molecular characterization of bacterial strains

The DNA of the two considered strains (PG1 and PG2), grown overnight in NBG on a shaking incubator (28 °C, 180 rpm), was extracted using the E.Z.N.A.® Bacterial DNA Kit. Primers 27F (GAGAGTTTGATCCTGGCTCAG) (Melníčáková et al. 2013) and 1495R (CTACGGCTACCTTGTTACGA) (Ventura et al. 2002) were used to amplify the 16S rRNA gene, using the protocol described in Supplementary Information file. The considered strains were also characterized for *nifH* encoding a nitrogenase as described in Supplementary Information file.

Seed sterilization and plant growth conditions

Seeds of two different pea genotypes commonly cultivated by Tunisian farmers were used: *cv.* Merveille de Kelvedon a medium-early, sweet and highly sought-after variety easy to grow, and *cv.* Lincoln, which is a medium-early variety, excellent for home gardens and organic production and it is adapted to the edapho-climatic conditions of Tunisia. Seeds were sterilized in 70% v/v EtOH for 60 s and in 2.5% v/v sodium hypochlorite (30 min), washed five times with sterilized water, put in Petri dishes on moist sterilized filter paper, and stored for 7 days at 25 °C (in the dark). Thirty Lincoln seedlings were transplanted to pots (0.7 L) containing sterilized quartz sand, while 30 Kelvedon and 60 Meraviglia d'Italia seedlings were transplanted to pots (1.4 L) containing sterilized quartz sand and coconut fiber (1:1 v/v). Plants were watered to compensate for evapotranspiration for one month, then twice *per* week with water and once a week with 50 mL ½ strength Hoagland solution (Hoagland and Arnon 1950), and they were grown in a greenhouse following the natural sun photoperiod (September–November 2022).

Bacterial inoculation in pea plants

After 28, 13 and 10 days after transplanting for Merveille de Kelvedon, Lincoln and Meraviglia d'Italia, respectively, plants were inoculated with the strains PG1 and PG2. Inoculum was prepared growing PG1 and PG2 for two days in NBG using a shaking incubator set at 26 °C (180 rpm). When PG1 and PG2 cultures reached the OD_{600} of 3.5, equivalent to approximately 1.9×10^9 and 1.2×10^9 CFU mL⁻¹, plants

were inoculated by pipetting 1 mL of culture in each pot at 1–2 cm depth, with pipette tips maintained at 45° to reach the root apparatus. Ten independent plants (representing the biological replicates) were prepared for each bacterial strain, in addition to other ten uninoculated control plants for genotypes Merveille de Kelvedon and Lincoln. Conversely, twenty independent biological replicates were used for each bacterial strain for Meraviglia d'Italia, in addition to twenty uninoculated control plants.

Salt application

After 32, 32, 23 days since transplanting, for Merveille de Kelvedon, Lincoln and Meraviglia d'Italia, respectively, 50 mL of NaCl (50 mM) starting solution was dispensed in each pot ($n = 5$ for Merveille de Kelvedon and Lincoln, $n = 10$ for Meraviglia d'Italia). Plants were subjected to salt stress watering them two times a week starting with a solution containing 100 mM NaCl and enhancing this concentration of 50 mM every three days to avoid salt shock, until reaching 200 mM NaCl as a stress condition (Pollastrì et al. 2018). After 15 days from transplanting (*i.e.*, 47, 47 and 38 days after transplanting for Merveille de Kelvedon, Lincoln and Meraviglia d'Italia, respectively) the experiment was stopped.

Biometrical and physiological measurements of plants

Before the beginning of the stress treatments, as well as during the stress period, biometric and physiological measurements were carried out. Height, node number, chlorophyll content by SPAD (Opti-Sciences) and stomatal conductance (g_s) with LI-COR 600 (Ecosearch) were measured. At harvest (*i.e.*, at the end of the stress treatment, when plants showed clear stress symptoms such as chlorosis), shoots were collected in liquid nitrogen and kept at -80°C for gene expression analysis and metabolic stress markers, while roots were oven-dried at 70°C for 48 h for biomass determination. For Meraviglia d'Italia plants, in addition to those analyses, shoot biomass was also determined using the

additional set of prepared replicates ($n = 5$). Pod number was recorded for all genotypes.

Biochemical analysis of *P. sativum* stress markers

Lipid peroxidation was evaluated through the malondialdehyde (MDA) content, resulting from the thiobarbituric acid (TBA) reaction (Heath and Packer 1968). Hydrogen peroxide (H_2O_2) amount was determined using the KI method (Loreto and Velikova 2001), while free proline levels were quantified using the ninhydrin reaction (Bates et al. 1973). Details have been reported in the Supplementary Information file.

Quantitative gene expression analysis in *P. sativum* leaves

Three biological replicates for each stress treatment (non-stressed, NS; salt stress, S) and for each inoculum (PG1, PG2, not inoculated control) were considered for the evaluation of gene expression. Total RNA was extracted from leaves (100 mg) of each biological replicate considered for gene expression ($n = 3$) utilizing the Spectrum Plant Total RNA extraction kit (Sigma-Aldrich) with slight modifications. RNA was quantified by NanoDrop 1000 spectrophotometer and then was treated with TURBO™ DNase kit following the routine DNase treatment (Thermo Fisher Scientific). DNA contamination absence was verified prior to proceed with the cDNA synthesis, by using pea *PsPP2A* specific primers in one-step RT-PCR (Knopkiewicz and Wojtaszek 2018) (Table 1). Total RNA was used to generate the cDNA, according to the SuperScript II Reverse Transcriptase® (Invitrogen) protocol using random primers. Each RT-qPCR reaction was conducted on a total volume of 10 μL , containing 1 μL diluted cDNA (dilution 1:2), 5 μL Power SYBR Green PCR Master Mix; Biorad), 3.6 μL of water and 0.2 μL of each primer (10 μM), using a 96-well plate. Primers are reported in Table 1. Reactions were performed using the Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories). Thermal cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles

Table 1 List of the primers used in the study and the putative function of the targeted genes

Putative function	Gene name	Forward primer	Reverse Primer	References
Elongation factor 1 alpha	<i>PsEFa1</i>	AGACCCTCGGACAAGCCCCT	ACCGGGCTTCACAACACCAGT	This work
protein phosphatase 2A	<i>PsPP2A</i>	CGGCTGCTGCGTGATAATG	TGGTAGAATATGCTGAATGGC TAGTT	Knopkiewicz and Wojtaszek (2018)
Superoxide dismutase	<i>PsSOD</i>	CCATCATAGGAAGGGCTGTTGT	CGTGACCACCTTTCCCAAGA	Gupta et al. (2021a)
Catalase	<i>PsCAT</i>	TCACAGAGGCACAGACTTGG	GACCTCCTCATCCCTGTGAA	Gupta et al. (2021a)
Ascorbate Peroxidase	<i>PsAPX1</i>	GAGATTACCGGTGGACCTGA	CTAAGCCCCATAGCCTTTCC	Gupta et al. (2021a)
Chlorophyll a/b binding protein	<i>PsCHL A/B</i>	CGGACCAGACCGTGTTAAGT	TGGATGACTTCAAGCTCACG	Gupta et al. (2021a)

at 95 °C for 15 s and 60 °C for 1 min. Pea target transcripts in leaves were quantified after normalization with two reference genes: *PsPP2A* encoding a protein phosphatase 2A (Knopkiewicz and Wojtaszek 2018) and *PsEF1a* coding for an elongation factor gene (NM_001427569.1). Three independent biological replicates were used (with the exception of control of salt stressed plants of genotype Meraviglia d'Italia for which two biological replicates were considered) as well as two technical replicates for each biological one. The candidate gene expression was calculated using the equation $2^{-\Delta\Delta CT}$ (Livak et al. 2001).

Statistical analysis

R software (version 4.1.1) was used for statistical analysis. Effects of pea genotype, applied stress, and inocula, on biochemical, biometric and physiological data at the end of the experiment were tested with analyses of variance (three-way ANOVA). Differences among treatments within the plant genotypes were statistically assessed using one-way ANOVA and a Tukey's HSD test, transforming data when necessary to fulfill ANOVA assumptions. A probability level of p -value < 0.05 was considered for all tests. Principal component analysis (PCA) was utilized for comparing both the biometric and physiological data, as well as the biochemical ones. R software (version 4.1.1) was utilized to calculate the Pearson correlation matrix. The Relative Expression Software Tool REST© 2009 v. 2.0.13 (Qiagen) (Pfaffl et al. 2002) was used for performing statistical analysis on gene expression data, using 0.05 as significance of p -value. To conduct the correlation analysis (Pearson correlation) between biochemical stress marker and gene expression (relative expression, ΔCT) data, a square root transformation was carried out to ensure normality and improve the linearity assumption. This analysis was conducted using the R package "PerformanceAnalytics". Specifically, the "chart. Correlation" function of this package was used. To assess the significance of the observed correlations, p -values were

calculated for each correlation coefficient, setting it at 0.05. The p -values were obtained using the built-in functionality of the "chart.Correlation" function.

Results

Characterization of bacterial strains

Biocontrol activity showed that six-days-old co-cultures of the phytopathogenic fungus *F. oxysporum* f. sp. *lentis* with isolates PG1 and PG2 showed on average $26.4 \pm 0.7\%$ reduction in fungal growth compared with controls (represented by the sole fungal colonies), with no significant differences among bacterial isolates. On the other hand, no growth reduction was recorded when *R. solani* was grown in the presence of PG1 or PG2. Molecular and biochemical characterization of the two selected strains was carried out by the 16S rRNA gene sequencing and evaluation of PGP traits. The sequencing of the PCR amplicons resulted in two sequences of 1368 bp (PG1) and 1388 bp (PG2) in length. These sequences were submitted in the GenBank database (accession number OQ919144 and accession number OQ919145 for PG1 and PG2, respectively) and compared against the NCBI database (16 March 2023). The two strains showed the highest identity with *Erwinia tasmaniensis*, followed by other species of *Erwinia* (Table 2). The phylogenetic tree confirmed the BLAST outputs, clustering the two strains close to *E. tasmaniensis* (Fig. 1). Based on PCR performed with primers for *nifH*, PG2 produced an amplicon between 300 and 400 bp, while PG1 did not. The amplicon was sequenced, showing 98.78% of identity with *Rhizobium* sp. KNb1 *nifH* (e-value of $2e^{-119}$) and submitted to NCBI GenBank (accession number OR083229). Tests in pure culture showed that both bacterial strains had the capacity to produce IAA and ACC deaminase activity and that PG1 showed the capacity to solubilize phosphate (Table 3). The ability of producing

Table 2 Best 5 hits based on PG1 and PG2 16S rRNA gene sequences obtained with primers 27F-1495R and compared against NCBI database RNA_type strains/16S_ribosomal_RNA

	Blast result	Coverage (%)	e-value	% identity	Accession number
PG1	<i>Erwinia tasmaniensis</i> Et1/99	100	0.0	99.12	NR_074869.1
	<i>Erwinia tasmaniensis</i> Et1/99	100	0.0	99.05	NR_042422.1
	<i>Erwinia endophytica</i> strain BSTT30	100	0.0	98.61	NR_148650.1
	<i>Erwinia billingiae</i> strain Billing E63	100	0.0	98.47	NR_104932.1
	<i>Erwinia billingiae</i> strain LMG 2613	100	0.0	98.49	NR_118431.1
PG2	<i>Erwinia tasmaniensis</i> Et1/99	100	0.0	99.14	NR_074869.1
	<i>Erwinia tasmaniensis</i> Et1/99	100	0.0	99.06	NR_042422.1
	<i>Erwinia endophytica</i> strain BSTT30	100	0.0	98.63	NR_148650.1
	<i>Erwinia billingiae</i> strain Billing	100	0.0	98.49	NR_104932.1

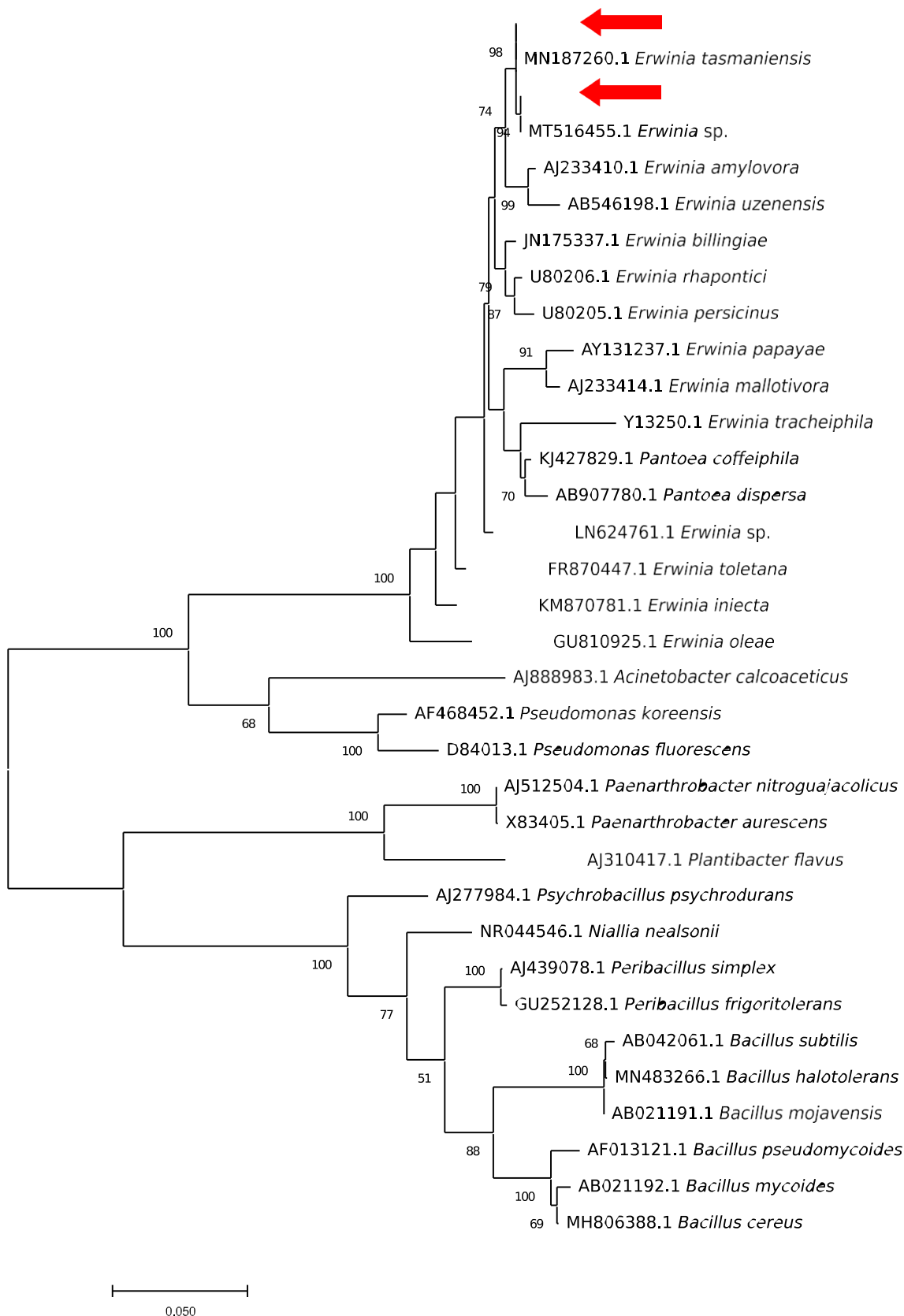


Fig. 1 Maximum parsimony bootstrap consensus tree obtained from 16S rRNA gene sequences. The numbers indicate bootstrap values and are given only for > 50%

Table 3 Results obtained by biochemical and physiological tests on bacterial strains PG1 and PG2 isolated from root nodules

Strain	IAA production (μg)	P solubiliza- tion halo (mm)	Residual ACC (%)	Fungal growth reduction activity in co-culture (% of control)	Bacterial growth at dif- ferent NaCl concentra- tions (%)	Bacterial growth at different PEG6000 concentrations (%)
				<i>F. oxysporum</i> <i>R. solani</i>	0.5 2.5 5.0 7.5 10.0	0 10 20 30 40 50
PG1	3.55 $\pm$ 0.07	31.2 $\pm$ 0.6	9.2 $\pm$ 0.6	25.7 $\pm$ 0.5	ND	+ + + + - + + + + -
PG2	4.08 $\pm$ 0.06	<5	12.1 $\pm$ 0.5	27.1 $\pm$ 0.4	ND	+ + + + $\pm$ + + + + - -

Data represent the average of three replicates $\pm$ SE. The growth on different concentrations of salt and PEG6000 are indicate with symbol + and -, where + indicates growth, - the absence of growth and $\pm$ stunted growth

extracellular NH_3 by both bacterial strains could be visually assessed by observing the development of the orange colour by surnatant-based reactions mixtures after the addition of Nessler's reagent. An apparent darker colour, suggesting higher NH_3 production, was detectable in the reaction mixtures obtained from the strain PG2. Unfortunately, the absorbance data recorded from both the original method and from the 96-well plates assays of supernatants from the two bacterial cultures were very variable among replicates, even when higher dilution levels (1:8 and 1:10, in place of 1:5) were used, and showed no significant differences among strains, whose extracellular NH_3 production ranged between 200 and 270 μM (not shown). In the salt stress test, PG1 was able to grow until a NaCl concentration of 7.5%, while PG2 could tolerate 10% NaCl, despite showing a reduced growth. Additionally, PG1 was able to grow on 40% PEG plates, while PG2 tolerated up to 30% PEG (Table 3).

***P. sativum* biometric and physiological parameter evaluation**

Biometric and physiological measures were taken during plant growth period, namely shoot height, node number and chlorophyll content, together with stomatal conductance, g_s . Then, after harvest the pod number and pod, root and, only for Meraviglia d'Italia, shoot dry weights were checked. The analysis of variance, considering genotype, inocula and stress, showed significant differences depending on the genotype for all the considered biometric and physiological parameters, and on the applied stress for height and physiological parameters (Table S1). The PCA using biometric and eco-physiological data, recorded at the end of the experiment, suggests that three genotypes performed in a variable manner (Figure S1). Considering Merveille de Kelvedon and Lincoln, genotypes employed in Tunisia, the PCA showed a differential response to salt (Fig. 2). A significant positive correlation was found between shoot height and node number, pod dry weight and root dry weight, shoot height and root dry weight, shoot height and pod dry weight, and node number and pod dry weight (Table S2). When data

from each genotype were analyzed independently, no significant differences were detected among the treatments for all the considered biometric parameters (Table S3). Concerning the physiological parameters, in Merveille de Kelvedon the chlorophyll content was significantly lower in salt stressed uninoculated and PG1-inoculated plants, compared with their non-stressed controls, while in the other two genotypes there were no statistical differences (Fig. 3). Pea plants belonging to Merveille de Kelvedon, grown without salt stress and treated with the strain PG2, had the tendency to show higher chlorophyll contents than those inoculated with PG1 (Fig. 3).

Relating to g_s values, in Merveille de Kelvedon and Lincoln the salt stressed plants showed significantly lower values than their controls, and a severe reduction of this variable under stress, irrespective of bacterial inoculation. Conversely, stomatal conductance (g_s) data obtained from Meraviglia d'Italia genotype were not significantly diverse between not stressed and stressed uninoculated and PG2-inoculated plants, while PG1 bacterial inoculation led a significant difference between not stressed and stressed plants (Fig. 3).

Biochemical analysis of *P. sativum* stress markers

The three-way ANOVA showed significant differences for each considered factor (genotype, applied stress, inoculation) (Table S4). The PCA carried out using biochemical parameters highlighted the differential responses of the three pea genotypes: while in Merveille de Kelvedon and Lincoln plants there was a separation between not stressed and stressed plants, in Meraviglia d'Italia this separation was less evident (Fig. 4). Considering each genotype separately, salt stress affected MDA and proline quantity in Merveille de Kelvedon, significantly increasing their values, while in Lincoln and Meraviglia d'Italia only slight differences were detected between stressed and not stressed plants (Table 4). Considering bacterial inoculation, salt stressed Merveille de Kelvedon plants inoculated with PG1 consistently showed the highest values of MDA, H_2O_2 and proline, while in the presence of PG2 only H_2O_2 content was significantly

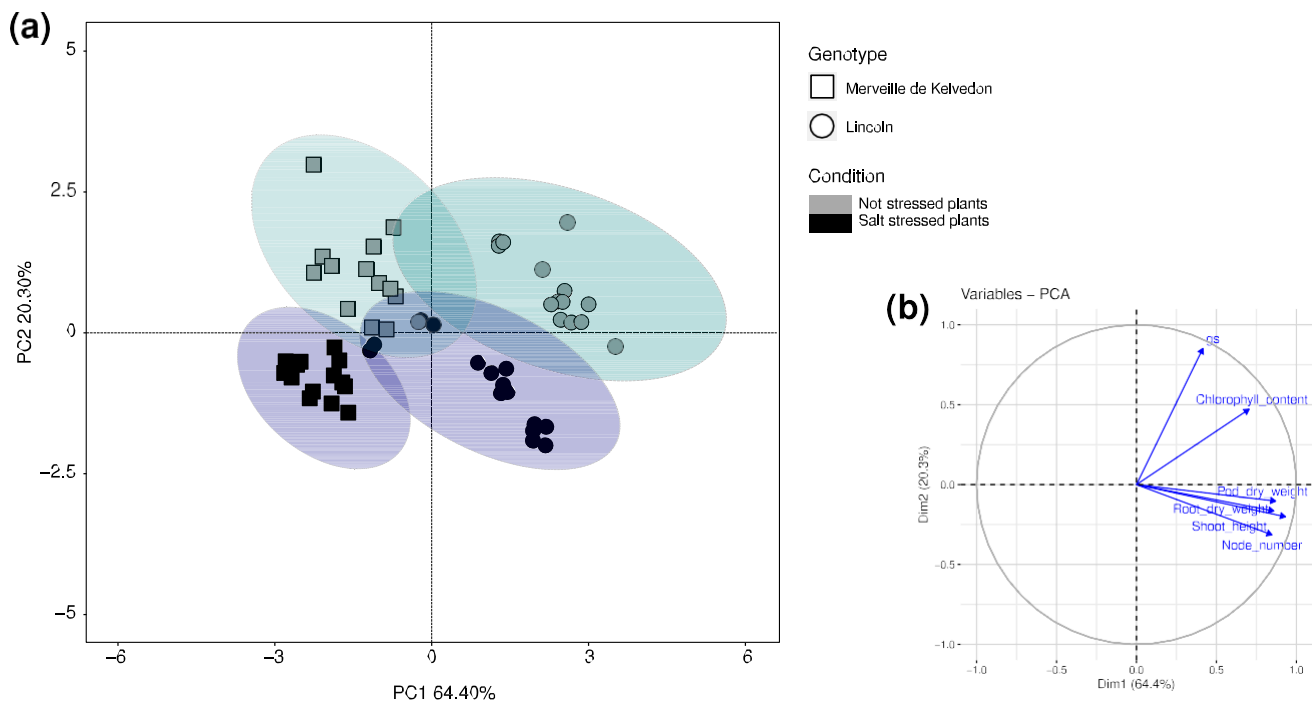


Fig. 2 Multivariate statistical analysis of physiological (chlorophyll content and g_s) and biometric (shoot height, node number, root and pod dry weight) data on Merveille de Kelvedon and Lincoln ($n = 5$). In (a), Principal component analysis (PCA) of samples; in (b), pro-

jection of variables, where angles are interpreted as correlations. The angle between two variable vectors represents the degree of correlation between them: adjacent (angle less than 90°) showed highly correlated variables, angle more than 90° showed uncorrelated ones

enhanced compared with both uninoculated stressed plants and not stressed ones (Table 4). Lincoln plants showed a largely different behavior. Significantly higher values of MDA were detected in not stressed PG1-inoculated plants and in stressed plants treated with PG2, compared with stressed uninoculated plants that showed the lowest value. H_2O_2 content was similarly low in stressed plants treated with PG2 and in not stressed uninoculated plants, while the highest value was found in stressed plants inoculated with PG1. Relating to proline, the highest value was found in stressed plants inoculated with PG1 and the lowest one in not stressed plants inoculated with PG2 (Table 4). In Meraviglia d'Italia, the lowest MDA value was recorded in stressed plants treated with PG2. Conversely, significantly higher values were detected in the other stressed plants, irrespective of inoculation, and in not stressed controls. No significant differences were found among treatments for H_2O_2 content, while the highest proline values were detected in stressed plants inoculated with PG1, which were significantly different from those found for all the other treatments (Table 4).

RT-qPCR results

To verify the plant response in the presence of salt, the expression of four genes putatively regulated by salt stress (Gupta et al. 2021a) was evaluated. The four genes include a

gene coding for a superoxide dismutase (*PsSOD*), a catalase (*PsCAT*), an ascorbate peroxidase (*PsAPX1*) and a chlorophyll a/b binding protein (*PsCHL A/B*). Relative expression of *PsAPX1* did not show differences among salt and/or bacterial treatments and control plants in all the genotypes, with the exception of Merveille de Kelvedon, where it was significantly up-regulated in stressed PG2-inoculated plants (Fig. 5). The transcription of *PsCAT* did not show differences among treatments in plants belonging to Merveille de Kelvedon genotype, while it was down-regulated in PG1-treated not stressed plants of Lincoln genotype, and up-regulated in stressed plants inoculated using the same isolate. In Meraviglia d'Italia genotype, this gene was upregulated in salt stressed controls (p -value 0.007), stressed PG1-treated plants and in PG2-inoculated plants, while it was down-regulated in PG1-inoculated plants (Fig. 5).

The gene *PsCHL A/B* was down-regulated in all the genotypes, although differently modulated by treatments. Significant down-regulation was detected in stressed Merveille de Kelvedon plants inoculated with PG1 and PG2, in all treatments of Lincoln genotype with the exception of non-stressed PG2-inoculated plants, and in PG1 treatments of both not stressed and stressed plants in Meraviglia d'Italia (Fig. 5). In Merveille de Kelvedon genotype, the *PsSOD* encoding gene was up-regulated only in stressed PG2-inoculated plants, while significant up-regulation in stressed

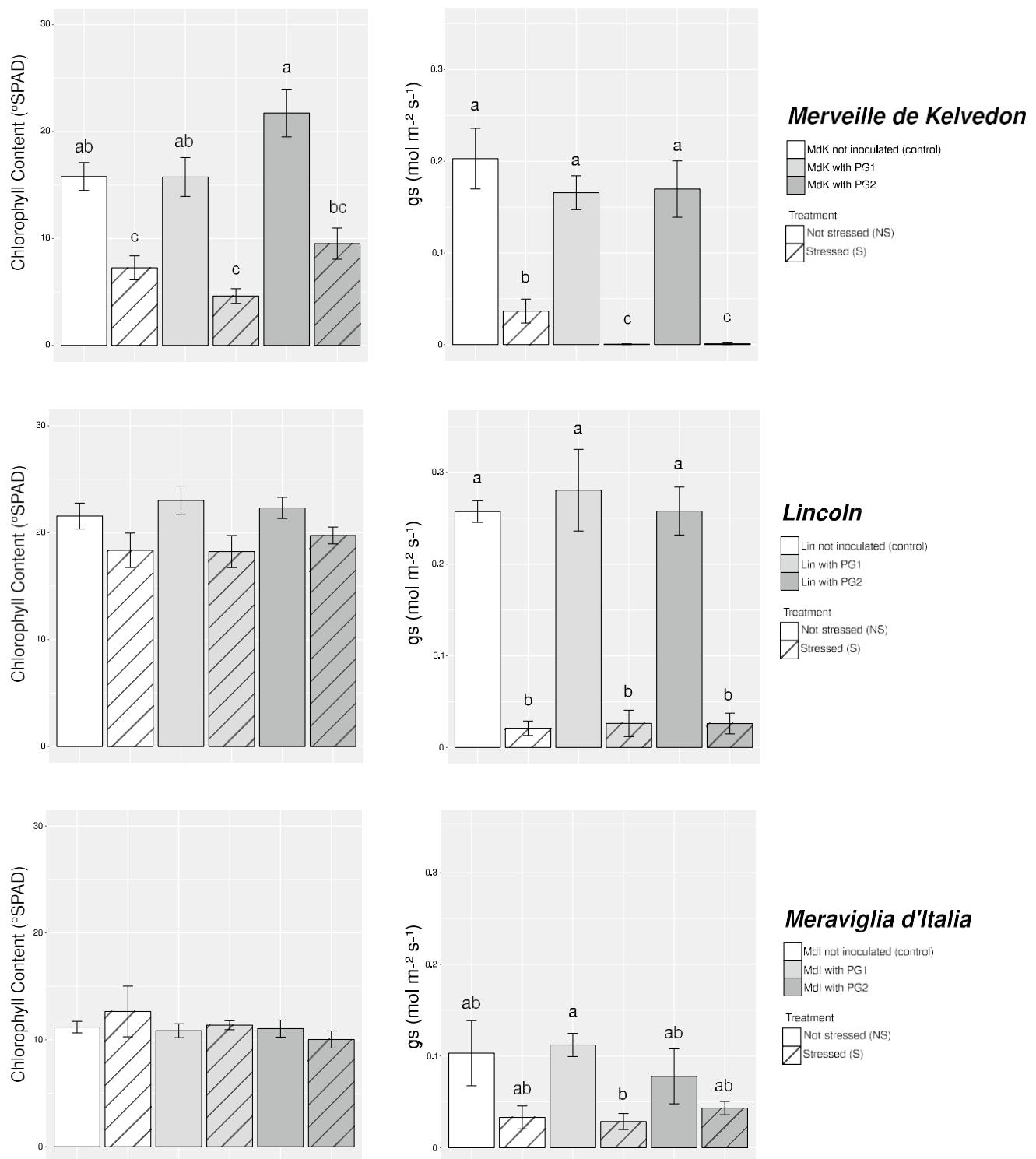


Fig. 3 Physiological data (chlorophyll content °SPAD and g_s mol m⁻² s⁻¹) in Merveille de Kelvedon (n = 5), Lincoln (n = 5) and Meraviglia d'Italia (n = 10). All data are expressed as mean ± SE (standard

error). Letters are plotted on the base of Tukey's test; different ones mean significant differences (p -value < 0.05). The absence of letters means absence of significant differences

controls and in PG1-inoculated and control stressed plants was detected in plants belonging to Lincoln and Meraviglia d'Italia genotypes, respectively (Fig. 5).

Gene expression and biochemical data correlation

Data obtained by gene expression and biochemical analyses

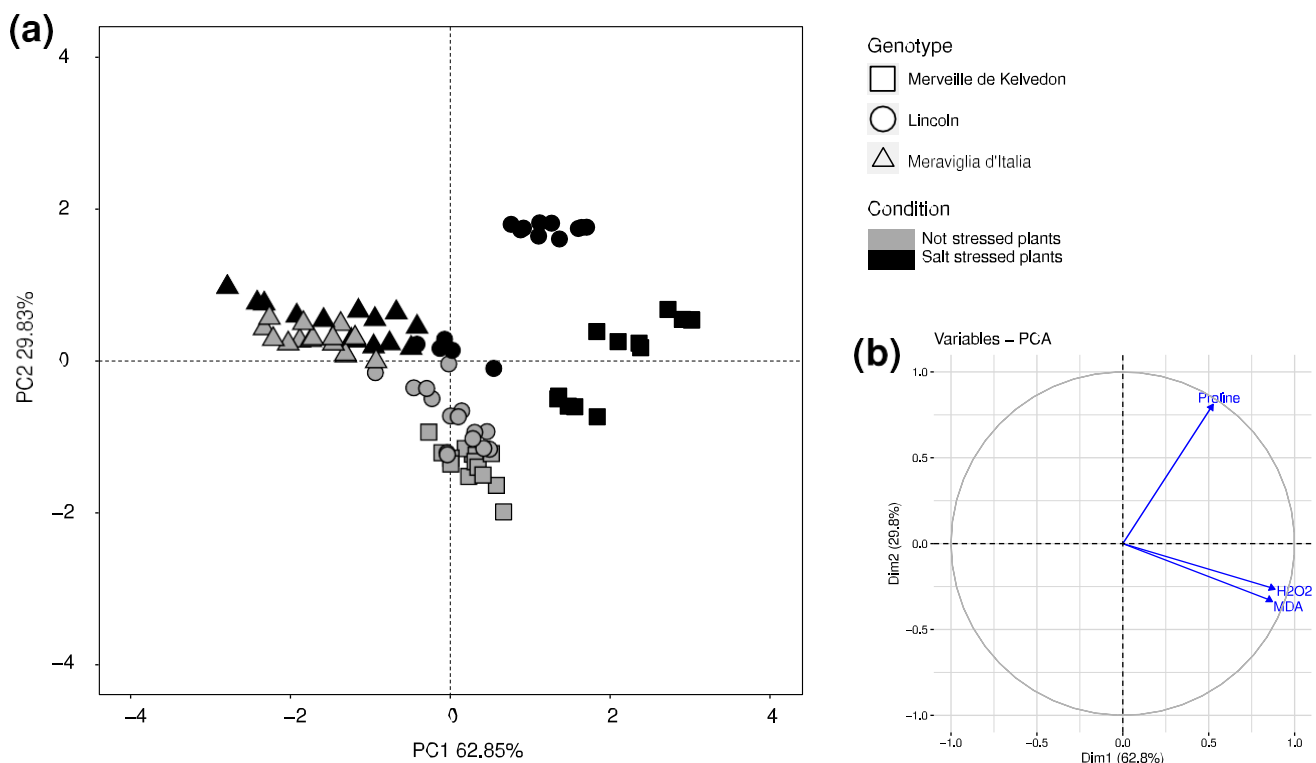


Fig. 4 Multivariate statistical analysis of biochemical data (MDA, H₂O₂, proline content) on the three genotypes (Merveille de Kelvedon, Lincoln and Meraviglia d'Italia). In (a), Principal component analysis (PCA) of samples; in (b), projection of variables, where

angles are interpreted as correlations. The angle between two variable vectors represents the degree of correlation between them: adjacent (angle less than 90°) showed highly correlated variables, angle more than 90° showed uncorrelated ones

were correlated, putting in evidence that in Merveille de Kelvedon and Lincoln nine correlations were detected, while in Meraviglia d'Italia only one. In detail, the significant positive correlations in Merveille de Kelvedon were between the relative expression of *PsSOD* and *PsCAT* and of *PsSOD* and *PsAPX1* and among the three stress markers (MDA, H₂O₂ and proline), while the relative expression of *PsCHL A/B* was significantly negatively correlated with the same stress markers. The significant positive correlations in Lincoln were between the relative expression of *PsSOD* and *PsCAT* and of *PsCAT* and *PsAPX1*, these three genes with proline, *PsSOD* with H₂O₂, H₂O₂ with proline, while the negative correlations were between the relative expression of *PsSOD* and *PsCHL A/B* and of *PsCHL A/B* with proline (Fig. 6). The only positive correlation in Meraviglia d'Italia was between the relative expression of *PsCAT* and proline.

Discussion

Plants can activate different mechanisms to protect themselves from stressful conditions, such as in high salinity (Raza et al. 2019). It has been widely reported that the

application of beneficial bacteria can increase salt tolerance in different crops such as tomato, chickpea, French bean, canola, wheat, rice, maize, potato, and pepper (Gupta et al. 2022; Orozco-Mosqueda et al. 2020). Gupta et al. (2021b) recently demonstrated in a pot experiment that *Bacillus subtilis* RhStr_71, *Bacillus safensis* RhStr_223, and *Bacillus cereus* RhStr_JH5 improved pea plant growth, increasing the abundance of osmoprotectant and antioxidant compounds and reducing oxidative stress level due to salt. In addition, combination of PGPB and mycorrhizal fungi has been documented to mitigate the negative salt effects, enhancing *Lathyrus cicero* plant growth (Gritli et al. 2022). Here, two bacteria isolated from Tunisian pea root nodules have been characterized molecularly and biochemically, and the effects of these strains on three pea genotypes in both optimal and salt stress conditions have been evaluated.

Characterization of bacterial strains

Sequencing of 16S rRNA gene allowed to verify that the two bacterial isolates both resulted to belong to *Erwinia* sp., phylogenetically close to *Erwinia tasmaniensis*. This species was first isolated from flowers and bark of apple and pear trees in Australia, showing that bacteria belonging to this species were non-phytopathogenic (Geider et al.

Table 4 Biochemical parameters (malondialdehyde, MDA; hydrogen peroxide, H₂O₂; proline) measured in three different pea genotypes inoculated or not with bacterial strains under salt stress (S) or control not stressed (NS) conditions

Genotype	Growth condition	Bacterial inoculation	MDA (nmol g ⁻¹ FW)	H ₂ O ₂ (μmol g ⁻¹)	Proline (μmol g ⁻¹)
Merveille de Kelvedon	NS	PG1	16.75 ± 1.28 c	0.06 ± 0.01 cd	7.78 ± 1.15 e
		PG2	16.48 ± 1.44 c	0.06 ± 0.01 cd	10.06 ± 0.88 de
		C	16.27 ± 1.26 c	0.05 ± 0.01 d	10.91 ± 0.93 d
	S	PG1	26.57 ± 1.39 a	0.09 ± 0 ab	133.5 ± 1.71 a
		PG2	19.97 ± 1.35 b	0.09 ± 0.01 a	72.18 ± 1.04 b
		C	20.55 ± 1.16 b	0.07 ± 0.01 bc	29.17 ± 1.47 c
Lincoln	NS	PG1	15.69 ± 0.74 a	0.05 ± 0 bc	15.58 ± 1.32 e
		PG2	14.59 ± 1.35 ab	0.06 ± 0.01 ab	10.78 ± 1.35 f
		C	13.77 ± 1.37 ab	0.04 ± 0 cd	18.93 ± 1.23 d
	S	PG1	14.32 ± 0.54 ab	0.07 ± 0.01 a	212.88 ± 1.14 a
		PG2	15.48 ± 1.05 a	0.04 ± 0.01 d	35.39 ± 1.89 c
		C	13.13 ± 0.7 b	0.06 ± 0.01 ab	171.46 ± 1.37 b
Meraviglia d'Italia	NS	PG1	9.12 ± 0.64 b	0.03 ± 0	20.61 ± 1.28 d
		PG2	8.58 ± 0.82 b	0.03 ± 0.01	21.63 ± 1.76 cd
		C	10.98 ± 0.83 a	0.03 ± 0.01	21.36 ± 0.78 cd
	S	PG1	12.15 ± 1.07 a	0.03 ± 0.01	38.95 ± 1.41 a
		PG2	6.71 ± 1 c	0.03 ± 0.01	23.67 ± 1.54 c
		C	11.51 ± 0.63 a	0.03 ± 0	26.66 ± 1.55 b

All data are expressed as mean ± SD (standard deviation). Different letters indicate significant differences (p -value < 0.05) within genotype. In Merveille de Kelvedon $n=5$ in CS, CNS and PG2NS, in the other treatments $n=4$; in Lincoln $n=5$ with the exception of the proline analysis were $n=4$ in CNS; in Meraviglia d'Italia $n=5$, with the exception of PG1S where $n=4$ and in proline CNS $n=4$

2006). The tests carried out by challenging tomato plants with the two isolates showed the absence of disease symptoms, confirming the non-pathogenicity of these bacterial strains, at least on two plant species (pea and tomato). The presence of non-pathogenic *Erwinia* species, among which *E. tasmaniensis* strains from different countries, was already reported (Sagar et al. 2018). Available genome sequences support these data, suggesting that non-pathogenic isolates lack the set of genetic factors needed for tissue invasion (Kube et al. 2010). Although some genetic traits involved in the induction of hypersensitive responses (HR) was still found in *E. tasmaniensis* strain Et1/99, able to start HR on tobacco leaves, no virulence on host apple and pear was detected (Kube et al. 2010; Palacio-Bielsa et al. 2012). The capacity of the two *E. tasmaniensis* strains to control the growth of a legume-derived isolate of *F. oxysporum* was detected in vitro, suggesting that screenings for antagonistic activity of nodule-associated microorganisms against microbial pathogens should be carried out, both in vitro and in vivo, to explore such potentially useful traits.

The molecular characterization of the bacterial strains was based on the search for the *nifH* gene (coding for nitrogenase), which is the most used marker gene for the identification nitrogen-fixing bacteria (Gaby and Buckley 2012). Here, its presence was detected in PG2, suggesting

the potential capability of this strain to reduce nitrogen into ammonia.

On the basis of the phenotypic characterization, PG1 may represent a strain with an applicative potential due to its capacity for solubilising phosphate, producing IAA and ACC deaminase, and to its ability to grow in presence of NaCl or PEG. The presence of these traits is common to other interesting PGPB such as *Planomicrobium* sp. MSSA-10 (Shahid et al. 2018), able to release organic acids in the rhizosphere resulting in increased P availability to plant roots, to regulate plant ethylene level and to produce root growth-promoting hormone. The observed features were also reported for another strain of *Erwinia* sp. (KP226572) that demonstrated to enhance wheat growth (Sagar et al. 2018). The bacteria producing ACC deaminase can help plant development, especially upon environmental stressful conditions (Ali et al. 2014). It was demonstrated that plants had higher levels of ethylene due to higher levels of ACC: in the presence of PGPB showing ACC deaminase activity, the ethylene levels decreased, leading to the production of α -ketobutyrate and ammonia (Gupta et al. 2022; Orozco-Mosqueda et al. 2020).

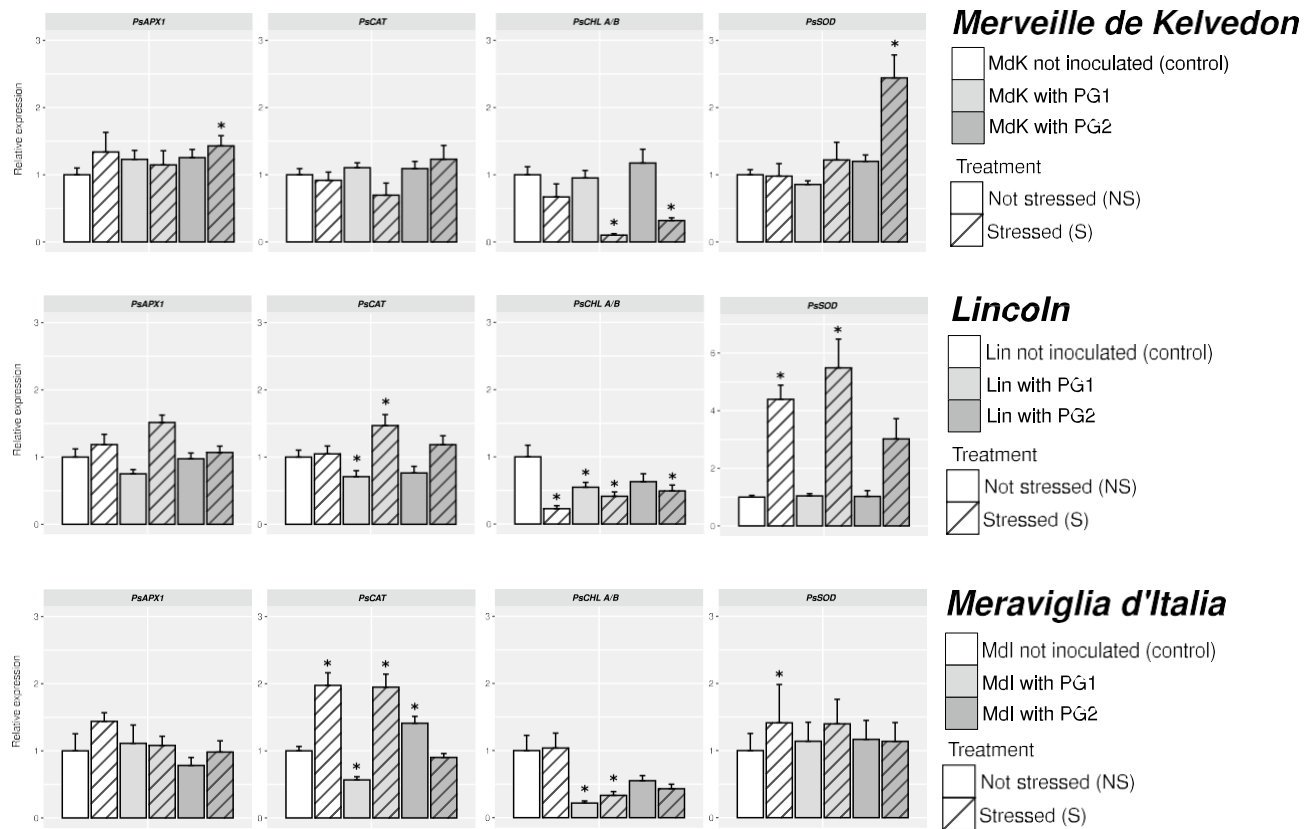


Fig. 5 Relative expression levels (Fold change $\pm$ SE), calculated following the equation $2^{-\Delta\Delta CT}$ of the four considered genes in Merveille de Kelvedon, Lincoln and Meraviglia d'Italia ($n = 3$, with the exception of stressed control in Meraviglia d'Italia where $n=2$). Asterisks

mean significant differences in comparison to control (non-stressed and uninoculated) plants, according to Pair Wise Fixed Reallocation Randomization Test © performed by REST© 2009

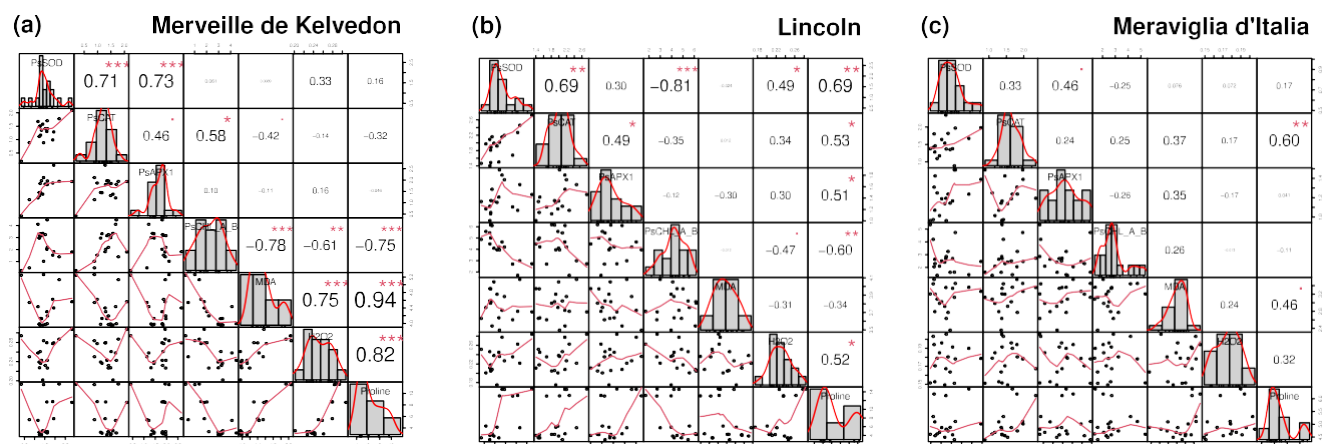


Fig. 6 Correlation matrices of biochemical and target gene expression data in the three genotypes. Matrix of data in Merveille de Kelvedon (a), Lincoln (b) and Meraviglia d'Italia (c). Each diagonal subplot shows the distribution of data of the considered variable as a grey histogram. Scatterplots of each pair of variables with a least-squares reference line (red) are also reported. The slope of red line corre-

sponds to the Pearson correlation coefficient. Numbers in the matrix represent the Pearson correlation coefficients (r). Font size used for correlation coefficients mirrors the degree of correlation. Red asterisks showed the p -value of the correlation ($=p<0.1$, $*=p<0.05$, $**=p<0.01$, $***=p<0.001$)

Effects of salt stress on biometric and physiological parameters of *P. sativum* plants

Although each genotype showed a different behavior, neither salt stress nor the presence of bacterial inoculation led to significant effects on growth parameters and root biomass. Previous studies showed that pea growth resulted negatively affected by salt treatments exceeding 50 mM and that variable responses could be detected among genotypes, depending on their tolerance (Shahid et al. 2011, 2012). A positive correlation between pod dry weight and root dry weight, and between pod dry weight and shoot height, was observed in Merveille de Kelvedon and Lincoln plants, suggesting that the tendency for more developed root and foliar systems can be associated with heavier pods. This result is in line with the fact that well-developed roots are generally expected to support nutrition, growth and development of the whole plant, as previously observed in other legume species (Gopalakrishnan et al. 2016).

Salt stress dramatically decreased the g_s values in Merveille de Kelvedon and Lincoln pea genotypes, independently on the inoculation, in agreement with studies showing that reductions in stomatal conductance, along with photosynthetic rates, are common plant response to salt stress, being these variables affected by Na^+/K^+ ratio and turgor decline (Hernández et al. 2000; Irshad et al. 2021). A less severe g_s variation was observed in Meraviglia d'Italia plants, suggesting that this genotype has a different stomatal behaviour with respect to the Tunisian cv. (Hochberg et al. 2018).

Stress marker evaluation

Three already known markers for stress, *i.e.*, MDA, H_2O_2 , and proline, were also evaluated. Large increases in MDA under salt stress were consistently detected in the Merveille de Kelvedon, particularly in the presence of the PG1 isolate; the same isolate also induced enhanced MDA levels in Meraviglia d'Italia stressed plants, whose uninoculated controls were unaffected by salt stress. Increase of MDA under salt stress was previously reported for different legume species, *i.e.*, pea, bean and lentil (Noreen and Ashraf 2009; Taïbi et al. 2016; Yasir et al. 2021). High concentration of MDA mirrors membrane lipid peroxidation, but it can also be correlated to an acclimation process signal, able to activate regulatory correlated to plant defense and growth, and to protect against oxidative stresses (Morales and Munné-Bosch 2019). Conversely, low concentrations of MDA have been related to the less active lipid peroxidation in salt-tolerance mechanisms. MDA levels were significantly reduced by inoculation with the isolate PG2 in stressed Meraviglia d'Italia plants, in the absence of changes in H_2O_2 content. As MDA may play different, and largely

unexplored, physiological roles, further studies are needed to reveal the impact of root-associated microorganisms on its accumulation. An increase of H_2O_2 concentration was detected in plants belonging to genotypes Merveille de Kelvedon and Lincoln under salt stress, according to studies already present in literature (Hernández et al. 2000; Irshad et al. 2021). Inoculation of salt stressed pea plants of both genotypes with PG1 increased H_2O_2 values, compared with uninoculated controls, while strain PG2 showed opposite effects on H_2O_2 levels, which were increased or decreased in salt stressed Merveille de Kelvedon or Lincoln pea plants, respectively. Thus, in Lincoln genotype the presence of PG2 bacterial strain might have reduced the plant stress status. In this regard, Neshat et al. (2022) suggested that the application of PGPR markedly decreased H_2O_2 levels, particularly under 100 mM salinity stress in canola plants. This reduction may be linked to the inhibition of Na^+ uptake through roots and a concurrent decrease in K^+ levels. Differential responses among genotypes and the lack of salt stress-related variations in H_2O_2 concentrations were previously reported for other pea cultivars (Noreen and Ashraf 2009). It is worth noting that, at high level, H_2O_2 is known to provoke oxidative damages to biomolecules, causing cell death, but at lower concentration it may work as a signaling molecule (Černý et al. 2018). The accumulation of such molecules in response to stress factors and microbial inoculants has proven highly dependent on plant and microbial identity and on their specific ability to modulate the multiple factors involved in stress response. Additionally, the regulation of this and other ROS depends on their generation and degradation, and on their neutralization rates by plant antioxidants. Results obtained on uninoculated plants of all pea genotypes confirmed that proline increases in the presence of osmotic stress. This amino acid plays an osmoprotective function, together with chaperone and antioxidant signal regulating function, whereas its specific role in response to stress in plants is still under debate (Szabados and Savoure 2010; Spormann et al. 2023). It is worth noting that Meraviglia d'Italia plants showed higher values of proline in not stressed conditions compared to the other two genotypes, and only a slight increase in proline content under stress. This result, together with the low MDA content detected in both not stressed and stressed Meraviglia d'Italia plants, may suggest a different response to salt stress for this genotype that harbors an enhanced basal level of proline compared to Merveille de Kelvedon and Lincoln. Additionally, g_s data of this genotype showed lower values in not stressed conditions compared to the other two genotypes. This reduced stomatal conductance could suggest a preventive and protective strategy against water loss by Meraviglia d'Italia plants. A recent work on the combined effect of drought and salinity on forage pea plants demonstrated that tolerant pea genotypes harbored high concentration of proline (Demirkol and

Yilmaz 2023). Other studies reported that the concentration of osmolytes and antioxidant enzymes in pea play an important role in salt tolerance potential (Farooq et al. 2017; Shahid et al. 2022). It is worth noting that the inoculation with the strain PG1 in Merveille de Kelvedon and Lincoln plant genotypes induced the largest accumulation of proline in response to salt stress (17- and 14-fold, respectively, over the not stressed Merveille de Kelvedon and Lincoln-inoculated plants). It has been documented that when salt stress mitigation measures are used, including the use of PGPB, proline levels often rise (Spormann et al. 2023). However, it is still under debate if proline accumulation results from changed redox balance and hormone metabolism or if it is a component of the tolerance-inducing process (Spormann et al. 2023). In our experiment, PG2 was less able to enhance proline accumulation under stress, as only a slighter increase in stressed plants was observed compared to inoculation with PG1, suggesting that PG2 inoculated plants may not need high proline accumulation. An explanation could be that these plants have an increase activity in proline degradation or that other osmotic solutes are involved in the response to stress (Szabados and Savaure 2010).

Gene expression and correlation analysis

To cope with the oxidative damage caused by salt, plants have developed defenses based on antioxidant enzymes such as SOD, POX, CAT (Hasanuzzaman et al. 2020). Here, expression levels of these genes were strictly dependent on the considered plant genotype-bacterial strain combination. In previous works, *P. sativum* plants inoculated with different isolates belonging to *Bacillus* or *Pseudomonas* showed an up-regulation of the genes coding for several antioxidant enzymes in comparison to uninoculated salt-stressed plants (Gupta et al. 2021a; Sofy et al. 2021). Here, inconsistent up-regulation of *PsCAT* and *PsSOD* was detected under salt stress/bacterial treatments in genotypes Lincoln and Meraviglia d'Italia, while *PsCHL A/B* was down-regulated or not significantly regulated, suggesting a diverse response to salt stress tolerance among pea genotypes, as already reported (El-Esawi et al. 2018; Khan et al. 2022). Correlating the gene expression and biochemical data, the highest number of significant correlations was detected in the two genotypes employed in Tunisia. In Merveille de Kelvedon, the relative expression of *PsSOD* was positively correlated to those of *PsCAT* and *PsAPXI*, suggesting a co-regulation of these scavenging genes. In Lincoln, the expression of *PsSOD* was also found to be significantly correlated with the expression of *PsCAT*, highlighting the co-regulation between these two functionally different genes. Additionally, always in Lincoln, a positive correlation between *PsSOD* expression level and

the amount of H_2O_2 could suggest a potential relationship between the expression of this gene and oxidative stress, considering that SODs are able to catalyze the dismutation of the superoxide ($O_2^{\cdot -}$) radical into O_2 and H_2O_2 (Bowler et al. 1994). A similar response to the treatments of these genes in both genotypes could be inferred. Nevertheless, looking at *PsSOD* and *PsCAT* expression on Merveille de Kelvedon and Lincoln inoculated plants, each pea genotype differentially responded to the two tested bacterial strains, with PG1 particularly affecting Lincoln stressed plants, while PG2 mainly impacted on Merveille de Kelvedon ones. In agreement with our data, a positive correlation between H_2O_2 level and proline content was previously found in plants subjected to environmental stress (Lee et al. 2022). The positive correlation between H_2O_2 and MDA in Merveille de Kelvedon could suggest a role of the peroxide in participating to lipid peroxidation and therefore to the membrane damages (Hajlaoui et al. 2009). Additionally, the correlation between MDA and proline in Merveille de Kelvedon could suggest that in presence of a cellular damage the plant adopts adaptive defensive mechanisms as osmoprotectants, as in celery under salt stress (Gao et al. 2023).

Chlorophyll a/b-binding proteins are reported to be involved in photosynthesis and to play a crucial role in capturing light energy (Bassi et al. 1997). The negative correlation between *PsCHL A/B* expression and the biochemical markers (MDA, proline, H_2O_2) in Merveille de Kelvedon, as well as between its expression and proline in Lincoln, suggested that when oxidative stress increases, and ROS rise, the expression of this gene decreases. This down-regulation may be a response to redirect cellular resources towards antioxidant defense mechanisms to cope with oxidative stress. It has been reported that reduced light absorption and increased anti-oxidative defense were associated with the down-regulation of chlorophyll a/b binding proteins (Munné-Bosch and Penuelas 2003; Xu et al. 2012). The down-regulation of *PsCHL A/B* in inoculated Merveille de Kelvedon and Lincoln plants under salt stress compared to uninoculated ones may also suggest that both PG1 and PG2 play a role in inducing this type of response. In Meraviglia d'Italia the only positive correlation was detected between *PsCAT* transcripts and proline; in upland drought tolerant rice varieties Lum et al. (2014) have already documented that their drought tolerance seemed to be correlated to activities of antioxidant enzymes, e.g., catalase, and an increase of proline content. A similar response can be hypothesized for a salt stress condition. The reduced number of correlations found in Meraviglia d'Italia could suggest that other types of responses are activated (Almeida et al. 2013), and that it could be differently affected by the applied stress.

Conclusion

The current study reveals that two non-pathogenic strains of *Erwinia* sp. are different in their PGP traits. By using an integrated approach, a picture of the pea plant status in three genotypes subjected to a salt stress condition was obtained and the role of the two bacterial considered *Erwinia* sp. strains has been highlighted. Results showed the relevance of plant genotype in determining the response to bacterial inoculants as well as the differences in the plant mechanisms activated to cope with the stress in the different plant/strain combination. Overall, this study emphasizes the importance of understanding the molecular and biochemical processes occurring in plant–microbe interactions at genotype level, and the influence on plant responses to environmental stresses. Further analyses are needed to clarify the behaviour of the three genotypes, such as the leaf water potential, and to verify the effects of the bacterial inoculation in field conditions, subjected by an increased environmental unpredictability due to the climate change scenario.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12298-024-01419-8>.

Acknowledgements This research was supported by the PRIMA RESCHEDULE project (Italian MUR DD 1293/2021) and the Tunisian-Moroccan bilateral project (20/PRD03). This study was carried out within the Agritech National Research Center and received funding from the European Union Next-Generation EU (PIANO NAZIONALE DI RIPRESA E RESILIENZA (PNRR) – MISSIONE 4 COMPONENTE 2, INVESTIMENTO 1.4 – D.D. 1032 17/06/2022, CN00000022). This manuscript reflects only the authors' views and opinions, neither the European Union nor the European Commission can be considered responsible for them.

Author contributions RB, BM: conceptualization; HI, CS, FB, MMEI, MNA, LO: isolation and biochemical bacterial characterization; HI, EZ, LG, MS, AC: greenhouse experiment; HI, EZ, FB, MS: molecular characterization and gene expression analysis; RM, GG, VF: stress markers characterization; EZ, LG, AC, FS: statistical analysis; HI, EZ: writing original draft; EZ, CS, FB, GG, MS, FS, VF, RB, BM: review and editing. All authors read and approved the final manuscript.

Funding Open access funding provided by Consiglio Nazionale Delle Ricerche (CNR) within the CRUI-CARE Agreement.

Data availability The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes

were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Abdelwahed S, Saadoui I, Kouidhi S et al (2022) A new pioneer colorimetric micro-plate method for the estimation of ammonia production by plant growth promoting rhizobacteria (PGPR). *Main Group Chem* 21:55–68. <https://doi.org/10.3233/MGC-210077>
- Acosta-Motos JR, Ortuño MF, Bernal-Vicente A, Diaz-Vivancos P, Sanchez-Blanco MJ, Hernandez JA (2017) Plant responses to salt stress: adaptive mechanisms. *Agronomy* 7:18. <https://doi.org/10.3390/agronomy7010018>
- Aeron A, Khare E, Jha CK et al (2020) Revisiting the plant growth-promoting rhizobacteria: lessons from the past and objectives for the future. *Arch Microbiol* 202:665–676. <https://doi.org/10.1007/s00203-019-01779-w>
- Ali SZ, Sandhya V, Venkateswar Rao L (2014) Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. *Ann Microbiol* 64:493–502. <https://doi.org/10.1007/s13213-013-0680-3>
- Almeida P, Katschnig D, de Boer AH (2013) HKT transporters—state of the art. *Int J Mol Sci* 14:20359–20385. <https://doi.org/10.3390/ijms141020359>
- Aloui M, Mahjoub A, Cheikh NB, Ludidi N, Abdelly C, Badri M (2022) Genetic variation in responses to salt stress in Tunisian populations of *Medicago ciliaris*. *Agronomy* 12:1781. <https://doi.org/10.3390/agronomy12081781>
- Bandeppa PS, Kandpal BK (2015) Evaluation of osmotolerant rhizobacteria for alleviation of water deficit stress in mustard. *Green Farming* 6:590. <https://doi.org/10.1016/j.plaphy.2019.08.018>
- Bassi R, Sandonà D, Croce R (1997) Novel aspects of chlorophyll a/b-binding proteins. *Physiol Plant* 100:769–779. <https://doi.org/10.1111/j.1399-3054.1997.tb00004.x>
- Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. *Plant Soil* 39:205–207. <https://doi.org/10.1007/BF00018060>
- Bhat MA, Kumar V, Bhat MA et al (2020) Mechanistic insights of the interaction of plant growth-promoting rhizobacteria (PGPR) with plant roots toward enhancing plant productivity by alleviating salinity stress. *Front Microbiol* 11:1952
- Bhat MA, Mishra AK, Jan S et al (2023) Plant growth promoting rhizobacteria in plant health: a perspective study of the underground interaction. *Plants* 1:629. <https://doi.org/10.3390/plant12030629>
- Bowler C, Van Camp W, Van Montagu M, Inzé D, Asada K (1994) Superoxide dismutase in plants. *Crit Rev Plant Sci* 13:199–218. <https://doi.org/10.1080/07352689409701914>
- Brescia F, Sillo F, Balestrini R, Sbrana C, Zampieri E (2023) Characterization of endophytic bacteria isolated from root nodules of lentil in intercropping with durum wheat. *Curr Res Microbiol Sci* 5:100205
- Cappuccino JGS (1999) Microbiology: a laboratory manual/James G., Cappuccino and Natalie Sherman (No. 576 C3). Benjamin-Cummings Pub Co.
- Černý M, Habánová H, Berka M, Luklová M, Brzobohatý B (2018) Hydrogen peroxide: its role in plant biology and crosstalk with

- signalling networks. *Int J Mol Sci* 19:2812. <https://doi.org/10.3390/ijms19092812>
- Demirkol G, Yilmaz N (2023) Morphologically and genetically diverse forage pea (*Pisum sativum* var. *arvense* L.) genotypes under single and combined salt and drought stresses. *Plant Physiol Biochem* 196:880–892. <https://doi.org/10.1016/j.plaphy.2023.02.041>
- Dworkin M, Foster JW (1958) Experiments with some microorganisms which utilize ethane and hydrogen. *J Bacteriol* 75:592–603. <https://doi.org/10.1128/jb.75.5.592-603.1958>
- El Idrissi M, Lamin H, Bouhnik O et al (2020) Characterization of *Pisum sativum* and *Vicia faba* microsymbionts in Morocco and definition of symbiovar *viciae* in *Rhizobium acidisoli*. *Syst Appl Microbiol* 43:126084. <https://doi.org/10.1016/j.syapm.2020.126084>
- El-Esawi MA, Al-Ghamdi AA, Ali HM, Alayafi AA, Witczak J, Ahmad M (2018) Analysis of genetic variation and enhancement of salt tolerance in french pea (*Pisum sativum* L.). *Int J Mol Sci* 19:2433. <https://doi.org/10.3390/ijms19082433>
- Etesami H, Li Z, Maathuis S, Frans JM, Cooke J (2022) The combined use of silicon and arbuscular mycorrhizas to mitigate salinity and drought stress in rice. *Environ Exp Bot* 10:4955. <https://doi.org/10.1016/j.envexpbot.2022.104955>
- FAOSTAT (2021) FAO statistical databases [WWW Document]. <https://www.fao.org/faostat/en/#data/QCL>. Accessed 12 June 2023
- Farooq M, Gogoi N, Hussain M et al (2017) Effects, tolerance mechanisms and management of salt stress in grain legumes. *Plant Physiol Biochem* 118:199–217. <https://doi.org/10.1016/j.plaphy.2017.06.020>
- Gaby JC, Buckley DH (2012) A comprehensive evaluation of PCR primers to amplify the *nifH* gene of nitrogenase. *PLoS ONE* 7:e42149. <https://doi.org/10.1371/journal.pone.0042149>
- Gao Y, Zhang J, Wang C et al (2023) Exogenous proline enhances systemic defense against salt stress in celery by regulating photosystem, phenolic compounds, and antioxidant system. *Plants* 12:928. <https://doi.org/10.3390/plants12040928>
- Geider K, Auling G, Du Z, Jakovljevic V, Jock S, Völksch B (2006) *Erwinia tasmaniensis* sp. nov., a non-phytopathogenic bacterium from apple and pear trees. *Int J Syst Evol Microbiol* 56:2937–2943. <https://doi.org/10.1099/ijs.0.64032-0>
- Gopalakrishnan S, Vadlamudi S, Samineni S, Sameer Kumar CV (2016) Plant growth-promotion and biofortification of chickpea and pigeonpea through inoculation of biocontrol potential bacteria, isolated from organic soils. Springerplus. <https://doi.org/10.1186/s40064-016-3590-6>
- Gritli T, Ellouze W, Chihoui SA, Barhoumi F, Mhamdi R (2019) Genotypic and symbiotic diversity of native rhizobia-nodulating red pea (*Lathyrus cicera* L.) in Tunisia. *Syst Appl Microbiol* 43:126049. <https://doi.org/10.1016/j.syapm.2019.126049>
- Gritli T, Boubakri H, Essahibi A, Hsouna J, Ilahi H, Didier R, Mnasri B (2022) Salt stress mitigation in *Lathyrus cicera* by combining different microbial inocula. *Physiol Mol Biol Plants* 28:1191–1206. <https://doi.org/10.1007/s12298-022-01205-4>
- Gupta A, Bano A, Rai S, Kumar M, Ali J, Sharma S, Pathak N (2021a) ACC deaminase producing plant growth promoting rhizobacteria enhance salinity stress tolerance in *Pisum sativum*. *3 Biotech* 11:514. <https://doi.org/10.1007/s13205-021-03047-5>
- Gupta A, Rai BA, Khanam A, Sharma S, Pathak N (2021b) Comparative evaluation of different salt-tolerant plant growth-promoting bacterial isolates in mitigating the induced adverse effect of salinity in *Pisum sativum*. *Biointerface Res Appl Chem* 11:13141–13154. <https://doi.org/10.33263/BRIAC115.1314113154>
- Gupta A, Mishra R, Rai S et al (2022) Mechanistic insights of plant growth promoting bacteria mediated drought and salt stress tolerance in plants for sustainable agriculture. *Int J Mol Sci* 23:3741. <https://doi.org/10.3390/ijms23073741>
- Hajlaoui H, Denden M, El Ayeb N (2009) Differential responses of two maize (*Zea mays* L.) varieties to salt stress: changes on polyphenols composition of foliage and oxidative damages. *Ind Crops Prod* 30:144–151. <https://doi.org/10.1016/j.indcrop.2009.03.003>
- Hasanuzzaman M, Bhuyan MHMB, Zulfiqar F et al (2020) Reactive oxygen species and antioxidant metabolism in plants under abiotic stress: revisiting the crucial role of a universal defense regulator. *Antioxidants* 9:681. <https://doi.org/10.3390/antiox9080681>
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Int Physiol Biochim Biophys* 125:189–198. [https://doi.org/10.1016/0003-9861\(68\)90654-1](https://doi.org/10.1016/0003-9861(68)90654-1)
- Hernández JA, Jiménez A, Mullineaux P, Sevilla F (2000) Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defences. *Plant Cell Environ* 23:853–862. <https://doi.org/10.1046/j.1365-3040.2000.00602.x>
- Hidri R, Barea JM, Mahmoud OM, Abdely C, Azcón R (2016) Impact of microbial inoculation on biomass accumulation by *Sulla carnosa* provenances, and in regulating nutrition, physiological and antioxidant activities of this species under non-saline and saline conditions. *J Plant Physiol* 201:28–41. <https://doi.org/10.1016/j.jplph.2016.06.013>
- Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. *Circ Calif Agric Exp Stn* 347:32
- Hochberg U, Rockwell FE, Holbrook NM, Cochard H (2018) Iso/ Anisohydry: a plant-environment interaction rather than a simple hydraulic trait. *Trends Plant Sci* 23:112–120. <https://doi.org/10.1016/j.tplants.2017.11.002>
- Ilahi H, Hsouna J, Ellouze W et al (2021) Phylogenetic study of rhizobia nodulating pea (*Pisum sativum*) isolated from different geographic locations in Tunisia. *Syst Appl Microbiol* 44:126221. <https://doi.org/10.1016/j.syapm.2021.126221>
- Irshad A, Rehman RNU, Abrar MM, Saeed Q, Sharif R, Hu T (2021) Contribution of rhizobium-legume symbiosis in salt stress tolerance in *Medicago truncatula* evaluated through photosynthesis, antioxidant enzymes, and compatible solutes accumulation. *Sustainability* 13:3369. <https://doi.org/10.3390/su13063369>
- Jia H, Xi Z, Ma J, Li Y, Hag C, Lu M, Zhang ZZ, Deng WW (2022) Endophytic bacteria from the leaves of two types of albino tea plants, indicating the plant growth promoting properties. *Plant Growth Regul* 96:331–343. <https://doi.org/10.1007/s10725-021-00779-5>
- Karnwal A (2009) Production of indole acetic acid by fluorescent *Pseudomonas* in the presence of L-tryptophan and rice root exudates. *J Plant Pathol* 19:61–63. <https://doi.org/10.4454/jpp.v9i11.624>
- Kebede E (2021) Contribution, utilization, and improvement of legumes-driven biological nitrogen fixation in agricultural systems. *Front Sust Food Syst* 5:767998. <https://doi.org/10.3389/fsufs.2021.767998>
- Khan MAH, Baset Mia MA, Quddus MA et al (2022) Salinity-induced physiological changes in pea (*Pisum sativum* L.): germination rate, biomass accumulation, relative water content, seedling vigor and salt tolerance index. *Plants* 11:3493. <https://doi.org/10.3390/plants11243493>
- Knopkiewicz M, Wojtaszek P (2018) Validation of reference genes for gene expression analysis using quantitative polymerase chain reaction in pea lines (*Pisum sativum*) with different lodging susceptibility. *Ann Appl Biol* 174:86–91. <https://doi.org/10.1111/aab.12475>
- Kube M, Migdoll AM, Gehring I et al (2010) Genome comparison of the epiphytic bacteria *Erwinia billingiae* and *E. tasmaniensis* with the pear pathogen *E. pyrifoliae*. *BMC Genom* 11:393. <https://doi.org/10.1186/1471-2164-11-393>
- Lee BR, La VH, Park SH, Mamun MA, Bae DW, Kim TH (2022) H₂O₂-responsive hormonal status involves oxidative burst

- signaling and proline metabolism in rapeseed leaves. *Antioxidants* 11:566. <https://doi.org/10.3390/antiox11030566>
- Li Z, Chang S, Lin L, Li Y, An Q (2011) A colorimetric assay of 1-aminocyclopropane-1-carboxylate (ACC) based on ninhydrin reaction for rapid screening of bacteria containing ACC deaminase. *Lett Appl Microbiol* 53:178–185. <https://doi.org/10.1111/j.1472-765X.2011.03088.x>
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>
- Loreto F, Velikova V (2001) Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. *Plant Physiol* 127:1781–1787. <https://doi.org/10.1104/pp.010497>
- Lum MS, Hanafi MM, Rafii YM, Akmar ASN (2014) Effect of drought stress on growth, proline and antioxidant enzyme activities of upland rice. *J Anim Plant Sci* 24:1487–1493
- Melničáková J, Derdákova M, Barák I (2013) A system to simultaneously detect tick-borne pathogens based on the variability of the 16S ribosomal genes. *Parasit Vectors* 6:269. <https://doi.org/10.1186/1756-3305-6-269>
- Mishra P, Mishra J, Arora NK (2021) Plant growth promoting bacteria for combating salinity stress in plants—Recent developments and prospects: a review. *Microbiol Res* 252:126861
- Mnasri B, Badri Y, Saïdi S, de Lajudie P, Mhamdi R (2009) Symbiotic diversity of *Ensifer meliloti* strains recovered from various legume species in Tunisia. *Syst Appl Microbiol* 32:583–592. <https://doi.org/10.1016/j.syapm.2009.07.007>
- Morales M, Munné-Bosch S (2019) Malondialdehyde: facts and artifacts. *Plant Physiol* 180:1246–1250. <https://doi.org/10.1104/pp.19.00405>
- Munné-Bosch S, Penuelas J (2003) Photo-and antioxidative protection, and a role for salicylic acid during drought and recovery in field-grown *Phillyrea angustifolia* plants. *Planta* 217:758–766. <https://doi.org/10.1007/s00425-003-1037-0>
- Nautiyal CS, Bhadauria S, Kumar P, Lal H, Mondal R, Verma D (2000) Stress induced phosphate solubilization in bacteria isolated from alkaline soils. *FEMS Microbiol Lett* 182:291–296. <https://doi.org/10.1111/j.1574-6968.2000.tb08910.x>
- Neshat M, Abbasi A, Hosseinzadeh A, Sarikhani MR, Dadashi Chavan D, Rasoulnia A (2022) Plant growth promoting bacteria (PGPR) induce antioxidant tolerance against salinity stress through biochemical and physiological mechanisms. *Physiol Mol Biol Plants* 28:347–361. <https://doi.org/10.1007/s12298-022-01128-0>
- Noreen Z, Ashraf M (2009) Assessment of variation in antioxidative defense system in salt-treated pea (*Pisum sativum*) cultivars and its putative use as salinity tolerance markers. *J Plant Physiol* 166:1764–1774. <https://doi.org/10.1016/j.jplph.2009.05.005>
- Orozco-Mosqueda MDC, Glick BR, Santoyo G (2020) ACC deaminase in plant growth-promoting bacteria (PGPB): an efficient mechanism to counter salt stress in crops. *Microbiol Res* 235:126439. <https://doi.org/10.1016/j.micres.2020.126439>
- Palacio-Bielsa A, Roselló M, Llop P, López MM (2012) *Erwinia* spp. from pome fruit trees: similarities and differences among pathogenic and non-pathogenic species. *Trees* 26:13–29. <https://doi.org/10.1007/s00468-011-0644-9>
- Passera A, Compant S, Casati P et al (2019) Not just a pathogen? Description of a plant-beneficial *Pseudomonas syringae* strain. *Front Microbiol* 10:1409. <https://doi.org/10.3389/fmicb.2019.01409>
- Peng M, Jiang Z, Zhou F, Wang Z (2023) From salty to thriving: plant growth promoting bacteria as nature's allies in overcoming salinity stress in plants. *Front Microbiol* 14:1169809. <https://doi.org/10.3389/fmicb.2023.1169809>
- Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 30:e36. <https://doi.org/10.1093/nar/30.9.e36>
- Pollastri S, Savvides A, Pesando M et al (2018) Impact of two arbuscular mycorrhizal fungi on *Arundo donax* L. response to salt stress. *Planta* 24:573–585. <https://doi.org/10.1007/s00425-017-2808-3>
- Raza A, Razaq A, Mehmood SS, Zou X, Zhang X, Lv Y, Xu J (2019) Impact of climate change on crops adaptation and strategies to tackle its outcome: a review. *Plants* 8:34. <https://doi.org/10.3390/plants8020034>
- Raza A, Tabassum J, Fakhar AZ et al (2022) Smart reprogramming of plants against salinity stress using modern biotechnological tools. *Crit Rev Biotechnol* 15:1–28. <https://doi.org/10.1080/07388551.2022.2093695>
- Sagar A, Thomas G, Rai S, Mishra RK, Ramteke PW (2018) Enhancement of growth and yield parameters of wheat variety AAI-W6 by an organic farm isolate of plant growth promoting *Erwinia* Species (KP226572). *Int J Environ Agric Biotechnol* 11:159–171. <https://doi.org/10.30954/0974-1712.2018.00178.21>
- Saghafi D, Delangiz N, Lajayer BA, Ghorbanpour M (2019) An overview on improvement of crop productivity in saline soils by halotolerant and halophilic PGPRs. *3 Biotech* 9:261. <https://doi.org/10.1007/s13205-019-1799-0>
- Sapre S, Gontia-Mishra I, Tiwari S (2022) Plant growth-promoting rhizobacteria ameliorates salinity stress in pea (*Pisum sativum*). *J Plant Growth Regul* 41:647–656. <https://doi.org/10.1007/s00344-021-10329-y>
- Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>
- Shahid M, Pervaz M, Balal R et al (2011) Effect of salt stress on growth, gas exchange attributes and chlorophyll contents of pea (*Pisum sativum*). *Afr J Agric Res* 6:5808–5816
- Shahid M, Balal R, Pervaz M et al (2012) Differential response of pea (*Pisum sativum* L.) genotypes to salt stress in relation to the growth, physiological attributes antioxidant activity and organic solutes. *Aust J Crop Sci* 6:828–838
- Shahid M, Akram MS, Khan MA et al (2018) A phyto-beneficial strain *Planomicrobium* sp. MSSA-10 triggered oxidative stress responsive mechanisms and regulated the growth of pea plants under induced saline environment. *J Appl Microbiol* 124:1566–1579. <https://doi.org/10.1111/jam.13732>
- Shahid S, Shahbaz M, Maqsood MF et al (2022) Proline-induced modifications in morpho-physiological, biochemical and yield attributes of pea (*Pisum sativum* L.) cultivars under salt stress. *Sustainability* 14:13579. <https://doi.org/10.3390/su142013579>
- Sharma S, Kulkarni J, Jha B (2016) Halotolerant rhizobacteria promote growth and enhance salinity tolerance in peanut. *Front Microbiol* 7:1600. <https://doi.org/10.3389/fmicb.2016.01600>
- Shen M, Jun Kang Y, Li Wang H, Sheng Zhang X, Xin Zhao Q (2012) Effect of plant growth-promoting rhizobacteria (PGPRs) on plant growth, yield, and quality of tomato (*Lycopersicon esculentum* Mill.) under simulated seawater irrigation. *J Gen Appl Microbiol* 58:253–262. <https://doi.org/10.2323/jgam.58.253>
- Sofy MR, Aboesidah AA, Heneidak SA, Ahmed HR (2021) ACC deaminase containing endophytic bacteria ameliorate salt stress in *Pisum sativum* through reduced oxidative damage and induction of antioxidative defense systems. *Environ Sci Pollut Res Int* 28:40971–40991. <https://doi.org/10.1007/s11356-021-13585-3>
- Spormann S, Nadais P, Sousa F et al (2023) Accumulation of proline in plants under contaminated soils - are we on the same page? *Antioxidants* 12:666. <https://doi.org/10.3390/antiox12030666>
- Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. *Trends Plant Sci* 15:89–97. <https://doi.org/10.1016/j.tplants.2009.11.009>
- Taïbi K, Taïbi F, Abderrahim LA, Ennajah A, Belkhdja M, Mulet JM (2016) Effect of salt stress on growth, chlorophyll content,

- lipid peroxidation and antioxidant defence systems in *Phaseolus vulgaris* L. South Afric J Bot 105:306–312. <https://doi.org/10.1016/j.sajb.2016.03.011>
- van Dijk M, Morley T, Rau ML, Saghai Y (2021) A meta-analysis of projected global food demand and population at risk of hunger for the period 2010–2050. Nat Food 2:494–501. <https://doi.org/10.1038/s43016-021-00322-9>
- Ventura M, Zink R (2002) Specific identification and molecular typing analysis of *Lactobacillus johnsonii* by using PCR-based methods and pulsed-field gel electrophoresis. FEMS Microbiol Lett 217:141–154. <https://doi.org/10.1111/j.15746968.2002.tb11468.x>
- Vidal-Valverde C, Frias J, Hernandex A et al (2003) Assessment of nutritional compounds and antinutritional factors in pea (*Pisum sativum*) seeds. J Sci Food Agric 83:298–306. <https://doi.org/10.1002/jsfa.1309>
- Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. In: IBP handbook 15, Blackwell, Oxford and Edinburgh
- Wang Q, Dodd IC, Belimov AA, Jiang F (2016) Rhizosphere bacteria containing 1-aminocyclopropane-1- carboxylate deaminase increase growth and photosynthesis of pea plants under salt stress by limiting Na⁺ accumulation. Funct Plant Biol 43:161–172. <https://doi.org/10.1071/FP15200>
- Wei X, Moreno-Hagelsieb G, Glick BR, Doxey AC (2023) Comparative analysis of adenylate isopentenyl transferase genes in plant growth-promoting bacteria and plant pathogenic bacteria. Heliyon 9:e13955. <https://doi.org/10.1016/j.heliyon.2023.e13955>
- Wei Z, Jousset A (2017) Plant breeding goes microbial. Trends Plant Sci 22:555–558. <https://doi.org/10.1016/j.tplants.2017.05.009>
- Xu YH, Liu R, Yan L et al (2012) Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in *Arabidopsis*. J Exp Bot 63:1095–1106. <https://doi.org/10.1093/jxb/err315>
- Yasir TA, Khan A, Skalicky M et al (2021) Exogenous sodium nitroprusside mitigates salt stress in lentil (*Lens culinaris* Medik.) by affecting the growth, yield, and biochemical properties. Molecules 26:2576. <https://doi.org/10.3390/molecules26092576>

Ringraziamenti

"The great thing about science is that it's true whether or not you believe in it"

Neil deGrasse Tyson

I primi ringraziamenti vanno ai miei tutor di Dottorato che mi hanno sostenuta in questo percorso e senza i quali questo percorso non sarebbe stato possibile. Un grazie sincero, quindi, alla Dottoressa Raffaella Balestrini e al Professor Quirico Migheli. Senza di voi questo percorso non sarebbe stato possibile e non c'è grazie più grande che possa dedicare. A tal proposito, vorrei ringraziare il Dottor Mauro Centritto, il cui contributo è stato fondamentale nel sostenerlo. Vorrei anche ringraziare una persona speciale, che mi ha vista crescere da studentessa indecisa durante i tirocini, sempre e comunque un po' indecisa, in questo percorso. Grazie quindi a Fabiano, grazie per aver creduto in me, per avermi spronata, per avermi bacchettata quando serviva ma, soprattutto, per avermi fatto capire cosa è per me realmente la ricerca. Mi hai insegnato tanto su come si analizzano i dati, ma soprattutto mi hai insegnato il potere del lato umano in questo lavoro. A Elisa grazie per la tua dolcezza e la tua disponibilità, mi hai sempre fatta sentire a casa, compresa e accettata. Grazie per tutte le esperienze che abbiamo condiviso, da quelle positive a quelle negative che mi hanno fatto capire che vorrei essere almeno un decimo della ricercatrice che sei tu. Grazie a Pi e Luca, due colleghi ma anche due amici che non pensavo di trovare. Non penso di poter racchiudere in qualche riga il valore che voi avete nella mia vita e quanto questo lavoro sia ancora più bello insieme a voi. Per lo stesso motivo, per lo stesso affetto incondizionato che provo, voglio aggiungere tutte le persone con cui ho costruito, più che un ambiente di lavoro, una casa. Grazie Buccio, Paolo e Giulia G., senza di voi non so cosa farei, a lavoro come nella vita. Siete la mia ancora e la mia bussola. Grazie anche a Marti, Giorgia, Mario, Ale Cice, Giulia L., Ceci, Fra Canuto, Fra Volpe, Ame, Marco, Bruni, Vale, Nico ed Eliana, siete quel gruppo di colleghi che capita una volta sola nella vita. Grazie anche a tutti i colleghi con cui ho iniziato questo percorso e che ora hanno preso strade diverse ma che rimangono sempre nel mio cuore. Grazie a Fra Bergese per aver creato, almeno per un po', il trio delle meraviglie con me e Pi. Abbiamo condiviso poco da colleghi ma continuiamo a condividere tanto da amici e di questo ti sono grata. Grazie alla girl squad: Flo, Fra Brescia, Monica, Sara e Silvietta, mi avete accolta, coccolata e mi avete insegnato tanto. Ovunque siate e ovunque finiremo, l'amore che mi avete dato rimarrà sempre dentro di me. Grazie a Daniele, per le mille chiacchierate, i mille consigli, i diecimila passaggi ma grazie soprattutto per essere diventato quello zio che non ho mai avuto. Grazie a Mic, sei stato un supporto inaspettato nella vita ma a cui non posso più rinunciare. Tu mi capisci sempre e condividiamo le stesse pare, gli stessi desideri e aspettative e non potrei volere compagnia migliore

in questa condivisione. In ultimo, grazie alla mia famiglia. Grazie alla mia famiglia che mi ha appoggiata nella scelta di questo percorso e che ha sempre pensato che ce l'avrei fatta.

Questo percorso è stato intenso, lungo e a volte stressante, ma lo rifarei altre mille volte.

Grazie per questa opportunità.

