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Negative plant-soil feedback in agroecosystems and natural plant communities: the role of soil chemistry, microbiota, and self-DNA

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Chapter 1: General introduction

Soil quality is one of the key factors controlling crop yield and health in an agroecosystem (Larkin, 2015). Soil quality is defined as the ability of soil to function within ecosystem boundaries to maintain biological productivity, preserve environmental quality, and promote plant and animal health (Doran & Parkin, 1994). Agricultural practices such as crop rotations, application of mineral fertilizers and organic amendments, tillage and use of agrochemicals significantly affect soil quality (Bastida et al. 2008; Wang et al. 2014). Soil quality results from the interplay of physical, chemical and microbiological factors, which in turn control water retention, soil structure and aggregate stability, organic matter dynamics, nutrient mineralization and soil pathogen suppression (Abiven et al. 2009). In recent decades, the spread of intensive agriculture worldwide has led to a significant decline in primary productivity, which has been associated with soil degradation (Bennett et al. 2012). Soil erosion, salinization (Naidu et al. 1995), soil compaction (Drewry et al. 2008), pollution by heavy metals and xenobiotics (Shen et al. 2005), decline in soil organic carbon (Johnston, 1986) and loss of beneficial microbiomes (Ibekwe et al. 2001) are all factors affecting soil quality. In this broad context, a special case of soil quality degradation is negative plant-soil feedback (NPSF).

As plants grow, they alter their soil environment, including nutrient availability and soil biota (Bennett et al. 2017; Fujii et al. 2018). These effects can affect seedling survival and growth as part of a process called plant-soil feedback (PSF), thus altering plant population and community dynamics (Bennett et al., 2017). NPSF is defined as the rise of negative conditions for plant vegetative and reproductive performance introduced into the soil by the plant itself (Bennett et al. 2012; Huang et al., 2013; Mazzoleni et al. 2007). This phenomenon is known in agronomy as "soil fatigue" (Schreiner & Sullivan, 1908). The NPSF has been shown to be strongly species-specific, i.e. it mainly affects individuals of the same species. In particular, sensitivity to NPSF decreases with increasing phylogenetic distance between species. Moreover, in the last three decades, researchers have recognized the importance of NPSF for the structure of natural plant communities and for the conservation of their biodiversity (Van der Putten et al. 2013). Adverse effects of NPSF have been described for several aspects of a plant's life cycle, including seed germination (Rice, 1984), seedling survival (Packer & Clay, 2000), growth (Miller, 1996), vegetative reproduction (Olff et al. 2000) and seed production. Most studies concerned the performance of individual species, while only a few addressed the indirect effects of NPSF on interspecific competitive interactions (Bonanomi et al. 2005).

NPSF is a complex, multifactorial phenomenon influenced by plant species, crop rotation, and soil management practices. In addition, environmental factors such as climate and

soil type can increase the complexity of the phenomenon. Three main hypotheses have been proposed to explain the mechanisms causing NPSF: soil nutrient depletion or imbalance (Howeler, 1991; Xiang et al. 2009); build-up of soil-borne pathogen and parasite populations (Manici et al. 2013; Packer & Clay, 2000), associated with a shift in soil microbial community composition (Klironomos, 2002; Kardol et al. 2007); and release of phytotoxic and autotoxic compounds during decomposition of crop residues (van de Voorde et al. 2012) or plant litter (Mazzoleni et al. 2015).

1.1 Soil nutrient depletion

The first hypothesis proposed to explain the NPSF and the resulting decline in plant production is that depletion or immobilization of nutrients in the soil causes deficiency in plants (Börner, 1960; Ehrenfeld et al. 2005). Plants can alter nutrient availability through nutrient depletion or changes in litter quality and nutrient cycling. Nutrient depletion usually leads to NPSF by limiting plant growth and can be exacerbated by a decrease in litter quality and thus nutrient input to the soil (Fujii et al. 2018). However, the effect of litter quality on PSF is complex. Slow-growing species with lower nutrient requirements can trigger a positive PSF by producing lower-quality litter, thereby reducing nutrient availability below the requirements of faster-growing species. Conversely, faster-growing species can trigger a positive PSF by producing high-quality litter that decomposes quickly, increasing nutrient availability and their competitive advantage over slower-growing species (Hobbie, 2015; Kulmatiski et al. 2017). However, most evidence from agroecosystems and natural plant communities does not support the nutrient depletion hypothesis.

A number of agronomic studies investigated the ability of nutrient fertilization to overcome NPSF, but most experiments showed that mineral fertilizers did not restore normal growth in diseased soils. For example, Zhou & Wu (2015) found that the content of macronutrients such as nitrogen, phosphorus and potassium in soil increased with the number of cucumber monoculture cycles. However, NPSF increased over time in mono cropping, and the effects were particularly dramatic after five production cycles. Moreover, Stinca et al. (2015) reported that the legume shrub *Genista aetnensis*, colonizing the bare lava flow of the Vesuvius Grand Cone was able, was able to establish an island of fertility under its canopy in a relatively short time by accumulating stocks of organic carbon, nitrogen, phosphorus, potassium, calcium, and magnesium, and improving soil hydrological properties. On the other hand, *G. aetnensis* seedlings were absent from the field under the canopy of conspecifics, and

bioassays in the greenhouse showed that seedling growth was inhibited in their own soil compared to the nutrient-poor substrate collected far from the canopy of conspecifics. Remarkably, coexisting phylogenetically unrelated plants thrived in the soil enriched with nutrients by *G. aetnensis* (Stinca et al., 2015). Evidence from both agricultural and natural ecosystems shows that the nutrient depletion hypothesis cannot be a satisfactory explanation for the development of NPSF.

1.2 Natural enemies and microbial shift

Natural enemies are widespread in soils and can contribute greatly to NPSF (Smith-Ramesh & Reynolds, 2017), with effects that can overwhelm nutrient-mediated PSF (Ke et al. 2015). NPSF was hypothesised to be due to the accumulation of pathogens in soil following the observation that soil sterilisation restores plant productivity in soils subjected to monoculture (Savory, 1966). In natural ecosystems, Packer & Clay (2000) provided clear evidence of NPSF driving the Janzen-Connell recruitment pattern for black cherry (*Prunus serotina*) in temperate forests of the USA. They observed widespread seedling failure among adult conspecifics, identifying *Pythium* sp. as the primary causal agent. Pathogens cause NPSF by reducing plant performance at different life stages, although they tend to affect younger, more susceptible plants (Sarmiento et al. 2017). Therefore, they are generally believed to be the cause of NPSF driven by soil biota (Kempel et al. 2018). It is notable, from the aforementioned studies, that most of the pathogens associated with NPSF are polyphagous fungi and oomycetes. Root herbivores, such as root-feeding nematodes, have received less attention but can also cause NPSF by feeding on the roots of young plants (Dias et al. 2018). However, species that use their resources for defence rather than growth should be less susceptible to NPSF caused by natural enemies (Cortois et al. 2016). Evidence that soilborne pathogens are consistently isolated from symptomatic plants supports the pathogenicity hypothesis, but the polyphagous nature of these pathogens does not fit the paradigm, as NPSF is highly species-specific. In fact, NPSF has been associated with species-specific pathogens in very few cases (Cesarano et al. 2017).

For a better understanding of the role of soil biota in NPSF, assessing the composition and changes of the completely microbial community is a necessary step. Recent studies have shown that the net effect of plant-soil feedback is the balance between beneficial and harmful microbes. Bennett et al. (2017), using 55 populations of North American trees, reported that soil collected beneath conspecifics showed NPSF for most of the species studied. In particular,

the type of mycorrhizal association with the plant species explained much of the variation in NPSF, with arbuscular mycorrhizal trees suffering a more intense NPSF than ectomycorrhizal trees. The authors suggest that ectomycorrhizal trees protect plant roots from soil-borne pathogens that accumulate under conspecifics. It is interesting to note that many crops suffering from severe NPSF are associated with arbuscular mycorrhizal fungi.

1.3 Phytotoxicity and autotoxicity

Many secondary chemicals, either released by root exudates or by decomposition of plant debris, can inhibit plant and microbial growth. Their production can have either negative or positive PSF, depending on whether these chemicals primarily affect conspecific (autotoxicity) or heterospecific (allelopathy) individuals. By definition, autotoxicity causes NPSF by inhibiting the growth of conspecifics. In some cases, autotoxic chemicals also inhibit mutualistic microbes and neutralize positive PSF (Zhou et al. 2018). Autotoxicity is common in agricultural systems and can occur in natural systems (Vincenot et al. 2017). Allelopathy inhibits the growth or mutualists of heterospecific plants and can cause positive PSF (da Silva et al. 2017). Allelopathy is more commonly studied in natural systems, although it is of growing interest for weed control in agricultural systems (Mariotte et al. 2018). The idea that NPSF might be caused by actively released toxins has been heavily criticized because such compounds are rapidly degraded by soil microbes into nontoxic molecules and therefore have limited effect under field conditions (Fitter, 2003).

In general, two non-exclusive hypotheses have been proposed to explain the inhibitory effect of plant residues on root growth: nitrogen immobilization by microbial competition (Hodge, 2004) and phytotoxicity by labile, low molecular weight organic compounds (Rice, 1984). The first hypothesis states that in the presence of decaying plant residues with a high C/N ratio, saprophytic microbes would compete with plants for nitrogen, resulting in temporary immobilization of this nutrient (Hodge et al. 2000). The second hypothesis assumes a direct negative impact on root growth caused by a broad spectrum of inhibitory compounds released early by the decomposing litter. In this context, microbial decomposition is of paramount importance as it influences the impact of plant residues on plant growth by modulating the relative abundance and activity of phytotoxic compounds (Cesarano et al. 2017). However, two main criticisms of the autotoxicity hypothesis have been raised. The first states that toxins from plant residues are rapidly degraded by microbial activity in the soil and become ineffective after a few weeks, whereas NPSF can persist in the field for months or even years; the second

states that many, if not all, organic compounds extracted from diseased soils and plant residues (Huang et al. 2013; Chen et al. 2015) exhibited general phytotoxicity, which is in contrast to the species-specificity of NPSF.

Alternatively, and in a more recent study, Mazzoleni et al. (2015) reported that fragmented extracellular self-DNA accumulated in litter during decomposition of conspecific residues in soil has species-specific inhibitory effects on various wild plants. These results not only provide a chemical basis for autotoxicity that must be considered in explaining NPSF, but also suggest an unexpected new functional role for exDNA in intra- and interspecific plant interactions at the ecosystem level (Carteni et al. 2016). Because exDNA is destroyed during soil sterilization by autoclaving or gamma irradiation, the known efficacy of this treatment to overcome NPSF cannot be used to distinguish exDNA toxicity from the pathogenic hypotheses. For this reason, the authors concluded that self- exDNA is a good potential candidate to explain NPSF (Mazzoleni et al. 2015). The hypothesis that exDNA may be involved in NPSF is intriguing, but further work is needed to validate this idea. Specifically, quantitative data on exDNA accumulation under field conditions and specific experiments are needed to confirm the inhibitory effect of purified conspecific DNA on seed germination and crop root growth (Barbero et al. 2016).

1.4 Aims & Scope

In this first chapter, i.e., the general introduction, I have provided an overview of the current knowledge on the mechanisms behind the negative plant-soil feedback processes, and I have given perspectives on the quantitative assessment of conspecific exDNA in order to assess its distribution and persistence in the soil.

This PhD thesis aims to increase the knowledge of plant-soil feedback processes in both agricultural and natural ecosystems. Specific objectives were investigated in the following chapters:

Second chapter: We investigated the phytotoxicity dynamics of litter decomposition of different plant species on the growth of *Trifolium repens* and *Triticum durum*. We then evaluated the impact of seed-associated endophytic fungi on the target species to different litter species with variable chemical properties. The hypothesis tested was that fungal endophytes would increase plant resistance to inhibitory effects of litter, based on their known beneficial effects on host plants. In this chapter, we first wanted to confirm the theory of phytotoxicity

from litter decomposition in soil and, in the meantime, test an underappreciated group of microbes, namely endophytic fungi, for plant response to released phytotoxicity.

Third chapter: Knowing that simultaneous colonization of a common host plant by endophytes and arbuscular mycorrhizal fungi (AMF) can affect not only the plant itself but also the next generation of the host plant via changes in the soil through plant-soil feedback processes, we investigated whether the interaction between fungal and bacterial endophytes in seeds and AMF affects the next generation of plants. Fungal seed endophytes have been reported to induce NPSF, and their association with AMF has been described as antagonistic. Therefore, we hypothesized that such association would increase the intensity of NPSF due to the reported antagonism on the host. However, we hypothesized that the association of bacterial endophytes in the seed with AMF would produce a positive PSF because each of these microbes has been shown to have multiple benefits for the host plant.

Fourth chapter: Before investigating the effect of the soil microbial community, including soil-borne pathogens and mutualists, on the generation of NPSF, we wanted to provide evidence that each plant species produces a specific microbial fingerprint under its canopy due to its specific litter decomposition and root exudates in the soil. Our hypothesis is that the chemistry of litter varies from plant to plant, resulting in a specific microbial fingerprint. It is hypothesised that this specific effect is due to the specific chemical properties of the shrubs' litter as it falls and decomposes, in addition to the plants' root exudates, resulting in different changes in soil chemistry and microbial composition. Therefore, the objective of this chapter was to provide basic information and novel insights into the environmental selection of soil microbial communities by each of the most abundant species-specific plants in a Mediterranean ecosystem.

Fifth chapter: In the context of this chapter, we observed an NPSF result in the field in the form of a Janzen-Connell (JC) distribution pattern. The JC hypothesis states that seeds are most likely to disperse at sites near their parent trees, where they are also most likely to be attacked by host-specific enemies such as insects and soilborne pathogens. Our initial field observations suggest that *Euphorbia dendroides*, a deciduous shrub, has a recruitment pattern consistent with JC distribution in a Mediterranean shrub area in southern Italy (Cape of Palinuro). For this reason, we first quantified whether JC distribution recruitment effectively occurs. In addition, because *Euphorbia* coexists with five woody species, we quantified recruitment under heterospecific shrubs as well. We then investigated the ecological causes for the observed

pattern. Specifically, we investigated whether soil chemistry and/or soil microbiota explained the observed pattern of seedling recruitment. We also explored differences in microclimate among shrub species by monitoring air temperature and light availability at different times of the year. We expected *Euphorbia* recruitment density to be positively correlated with soil fertility, light, temperature buffer effect, and beneficial soil microbes, while negatively correlated with soilborne pathogens.

Sixth chapter: In this chapter, we transferred the challenge this time from natural to agricultural ecosystems. Specifically, we examined how eight crops promote or inhibit conspecific and heterospecific growth through changes in the soil. The exclusivity of the work is because we used a large number of plants with a high combination number during the second round of growth, i.e., the reaction phase. More importantly, we used the entire soil history for the response phase and not just 10% conditioned soil inoculum as most studies did. We assumed that plant communities in the conditioning phase would influence soil chemical properties and soil biotic composition, and we expected that soil biota would influence the establishment of future plant communities in the response phase. Therefore, the objective of this chapter was to test the effects of the different soil legacies established by each plant species during the conditioning phase on the chemical and microbial properties of the soil and, consequently, on the growth of conspecifics and heterospecifics during the response phase.

Seventh chapter: In this chapter, we conditioned *Arabidopsis thaliana* over a long period to affect soil biotic and abiotic properties through both root exudates and litter decomposition. After the conditioning phase, the plants were removed and the soil was subjected to four different treatments, namely sterilization by autoclaving, washing with tap water, addition of 10% activated carbon and untreated control. Then, another growth cycle was started. After the response phase, the plant biomass grown in each of the four treatments was recorded, as well as the soil chemical properties and microbiota, using Shotgun sequencing. In addition, for the first time, we quantified *A. thaliana* self-DNA in each of the treated soils as well as in the preconditioned soil using chloroplast rbcL DNA primers. The purpose of this study was to detect the accumulation of self-DNA in the soil during the conditioning phase and to show that this exDNA is associated with the increase in NPSF. In addition, we wanted to test whether soil sterilization leads to confounding in clarifying the mechanisms behind the NPSF, as this treatment affects both soil microbial communities, particularly soil pathogens, and soil exDNA. Furthermore, we wanted to test the theory that activated carbon accumulates exDNA in the soil, thus amplifying the effects of the NPSF.

2 Chapter 2: Fungal endophytes affect *Trifolium repens* and *Triticum durum* plant response to different leaf litter with contrasting chemical traits

2.1 Abstract

Plant litter decomposition is a crucial process of nutrient cycling within ecosystems. However, many studies have shown that, apart from its several beneficial effects, organic matter decomposition can be disadvantageous to seed germination, seedling growth, and physiological activity of plants. Litter decomposition was reported to affect both plants and their associated soil microbial communities. The aim of this work was to test the relationships between seed-associated endophytic fungi on the either positive or negative plant's response to different litter types. Leaf material of four species was collected and used in a decomposition experiment inside a growth chamber for 120 days. The plant growth experiment was set in a greenhouse using *Trifolium repens* and *Triticum durum* with and without their associated endophytic fungi in the presence of the different litter species at two decay levels (fresh litter and after 120 days of decomposition). Results demonstrated that fresh litter exerted a strong inhibition effect on the plant total biomass when compared to decomposed litter. Moreover, seed-associated endophytic fungi enhanced the inhibitory effect of litter in the observed experimental conditions. The removal of seed-associated endophytic fungi improved the capacity of tested plants to resist to litter inhibitory effect.

Keywords: Endophytic fungi; Inhibition effect; Litter decomposition; Seedling growth; Soil microbial communities.

2.2 Introduction

Litter decomposition is a fundamental ecological process for sustaining life on earth, as it is maintaining ecosystems functions and nutrient cycling (Berg and Laskowski 2005). Broadly defined, decomposition consists of the breaking-down of organic matter into CO_2 and nutrients via physical, biological and chemical means (Aerts 1997, Krishna and Mohan 2017). During these processes, a large fraction of carbon is released into the atmosphere while a smaller fraction is transformed into humus substances, which accumulates in the soil for a long time and reused by microbes and plants (Berg and McClaugherty 2014).

The rate of the organic matter decay is affected by litter quality (Meentemeyer 1978, Manzoni et al. 2010, Bonanomi et al. 2013), and climatic factors (Aerts 1997). Litter decomposition is responsible for generating a vital part of the nutrient budget on the scale of ecosystems and the whole biosphere (Vesterdal 1999, Krishna and Mohan 2017). However, many studies have demonstrated a detrimental effect of decomposing litter on seed germination, seedling survival, and plant growth (Van der Putten et al. 1997, Bonanomi et al. 2005, Zhang et al. 2015). Allelochemicals, mostly referable to 'secondary metabolites', have been reported to affect neighbouring plant individuals and soil microbial communities including bacteria, nematodes, pathogens and mycorrhizal fungi (Schenk et al. 1999, Souto et al. 2000, Shaukat et al. 2002). Litter decomposition produces various organic compounds that are subjected to several physical, chemical, and biological processes in the soil, such as sorption and polymerization by soil organic matter and clay minerals (Makino et al. 1996), and chemical transformation by microorganisms (Blum et al. 1999). These changes affect both the composition and quantity of allelochemicals, which may either increase or decrease the phytotoxicity of decomposing plant litter (An et al. 2001). Investigations about phytotoxicity dynamics have demonstrated that, generally, most severe inhibition effects have been observed in early stages of decomposition, followed by decreases in phytotoxicity (Cochrane 1948, Jäderlund et al. 1996, Bonanomi et al. 2006). Recently, Mazzoleni et al (2015) showed that while the early litter phytotoxicity, mostly acting on heterospecific plants, is typically related to labile compounds, the long-term self-toxicity might build-up during decomposition due to accumulating of self-DNA of the same plant species in the decaying organic materials.

Since the 1980s, an increasing number of studies have demonstrated that soil microorganisms are the main decom- posers by consuming over ~95% of plant detritus, leaving the slight proportion of ~5% to soil animals (Berg and Laskowski 2005, McGroddy et al. 2004,

Cleveland and Liptzin 2007). Among the soil microbial population, fungi are the leading decomposers and have more than 75% greater potential to crumble organic matter than other microorganisms (Kjoller and Struwe 1992, Krishna and Mohan 2017). One notable subgroup in the fungal kingdom is the endophytic fungi.

Fungal endophytes have been well studied over the past few decades (Hyde & Soytong, 2008). Endophytic fungi are defined as plant associated fungi that colonize and live, during a specific phase of their life cycle, within a part of a plant without causing any apparent damage or disease to their host (Hardoim et al. 2015, Puri et al. 2016). Endophytic fungi have been reported as natural residents within several host plants (Saikkonen et al. 1998, Suryanarayanan 2013, Bamisile et al. 2018). Different species of endophytes can be enclosed in a single part of the plant (leaf, stem, seed or root) (Cherry et al. 1999, Vega et al. 2008, Bamisile et al. 2018). In general, fungal endophytes are known to be beneficial, protecting their host plants from pathogens (Campanile et al. 2007), by producing secondary metabolites (Schulz et al. 1999), and cell wall-degrading enzymes (Cao et al. 2009), or by inducing systemic resistance (Vu et al. 2006). Moreover, they are capable of protecting their host against several abiotic stresses as well, such as drought (Elmi and West 1995), salinity, nutrient depletion (Malinowski et al. 2000), flooding (Giordano et al. 2009), and thermal stress (Redman et al. 2002). On the other hand, few species have been reported as pathogens, causing disease to the host, after a period of latency (Mayerhofer et al. 2012, Kia et al. 2017, Sikora et al. 2007). Other species are considered neutral without implying benefits nor damages (Sikora et al. 2008). In addition to their role within plants, many endophytes can survive and grow as saprophytes in soils (Peay et al. 2016), and include species that are decom- posers of plant material (Song et al. 2017). However, the exact conditions under which most endophyte species function remain largely unknown (Newsham 2011).

Research on the interaction between allelopathy and endophytic fungi has been sufficiently explored. Yue et al (1998) revealed, for instance, the detoxification of the allelochemicals Benzoxazolin-2-one (BOA) and 6-methoxybenzoxazolin- 2-one (MBOA) produced by corn to N-(2-hydroxyphenyl) and N-(2-hydroxy-4-methoxyphenyl) malonamic acids, respectively by the endophytic fungus of corn *Fusarium moniliforme* J. Afterwards, Zikmundová et al (2002) studied the bio- transformation and detoxification of two allelochemicals BOA and HBOA by four endophytic fungi isolated from *Aphelandra tetragona*. However, the effect of these endophytic fungi on the response of plants to litter decomposition and associated phytotoxic compounds has never been tested. Moreover, litter

having different chemical traits, depending on plant type and decomposition age, could have variable effects on plants and seed endophytic fungi. Therefore, the aim of this work is, firstly, to test the effect of different litter type on the response of two target species, *Trifolium repens* L. and *Triticum durum* L. Secondly, we assessed the impact of seed-associated endophytic fungi on the target species response to four litter species having variable chemical traits. The tested hypothesis was that fungal endophytes would enhance the resistance of plants to litter inhibitory effect, based on their known beneficial effects reported on the host plants.

2.3 Material & Methods

2.3.1 Plant litter collection

Litter of four species of different functional groups were selected including a forb (*Hedera helix* L.), a deciduous tree (*Fraxinus ornus* L.), an evergreen tree (*Quercus ilex* L.), and a deciduous, nitrogen fixing tree (*Alnus glutinosa* L.). The species, belonging to different functional groups, were selected to evaluate the effect of a range of chemical traits on the target plant response with and without associated endophytic fungi. Litter samples were taken from vegetation types of both Mediterranean (*H. helix* and *Q. ilex*) and temperate environments (*A. glutinosa* and *F. ornus*) (Campania region, Southern Italy). For each species, the litter was collected by placing nets under the canopy. The fallen leaves were periodically collected, transferred to laboratory, dried in a ventilated chamber (40°C for 10 days), chopped with scissors (size < 3 cm), and stored at room temperature (Bonanomi et al. 2011).

2.3.2 Decomposition experiment

In open fields, litter decomposition essentially relies upon organic matter quality, water availability and temperature (Berg & McClaugherty, 2014). In order to focus only on litter quality effects, the decomposition experiment was carried out under controlled conditions. The litter decomposition experiment was performed in a growth chamber with optimal conditions of water availability and temperature, since the litter was watered every 7 days with sterile distilled water at holding capacity, and the temperature was 18 ± 2°C at night and 24 ± 2°C during the day. Dry leaf litter (100 g for each species in 3 replicates) was placed inside plastic trays (size 30 cm × 50 cm × 50 cm). A microbial inoculum, obtained by mixing 10 g of soil from the fields of litter collection (top 10 cm) and 90 g of water was added improve the outset and the maintenance of the decomposition process (Bonanomi et al. 2011). The microbial inoculum was sprinkled over the litter trays. Decomposed litter was collected 120 days of incubation. Eight samples were obtained (4-litter species × 2 sampling dates, 0 and 120 days

of incubation). Litter was air dried in paper bags in a ventilated chamber at 40°C until a constant weight was reached.

2.3.3 Litter chemical analyses

All litter samples were characterized for total C and N content by flash combustion of micro-samples (5 mg of litter) using an elemental analyser (Flash EA2000 Thermo). The relative content of acid-detergent hydrolysable fraction (thereafter indicated as labile C) was determined by mild acid hydrolysis with 0.5M H_2SO_4 and the detergent cetylt-rimethyl-ammonium (20 g l−1). Proximate cellulose was quantified as hydrolysable fraction after an extreme sulphuric acid treatment (loss due to 72% H_2SO_4 for 3 hours), and proximate lignin as the unhydrolyzable fraction (loss upon ignition after the above sulphuric treatment (Gessner 2005). All carbon fractions are presented as ash-free dry mass.

2.3.4 Effects of the fungal endophytes on plant response to litter

To test the effect of seed-associated endophytic fungi of two target plants (*T. repens* and *T. durum*) in response to litter of different species and ages, we conducted a greenhouse experiment in the period between March and June 2018. Target species used in this experiment were chosen due to their short life span, being agricultural annual, and because belong to different functional types, i.e., a grass and a nitrogen fixing species. Moreover, test plants are well known to suffering from soil sickness caused by litter phytotoxicity (Cesarano et al. 2017). Therefore, the aim was to test the effect of seed associated fungi in modulating the soil sickness problem for agricultural plants.

T. repens and *T. durum* plants were grown in 54 pots with 2 plants in each. A total of 27 pots were planted with seedlings containing endophytic fungi (hereafter indicated as EF+) and other 27 without their natural fungal endophytes (hereafter indicated as EF-), in the presence of litter of four species (*H. helix*, *A. glutinosa*, *F. ornus* and *Q. ilex*) at two ages (fresh 0 d and decomposed for 120 d). The control was without litter addition while in the litter treatments the organic matter was incorporated at rate of 1% by weight. Overall, we obtained 18 experimental treatments (8 litter types plus untreated control, with EF+ and without EF- endophytic fungi) for a total of 108 pots (Fig. 1).

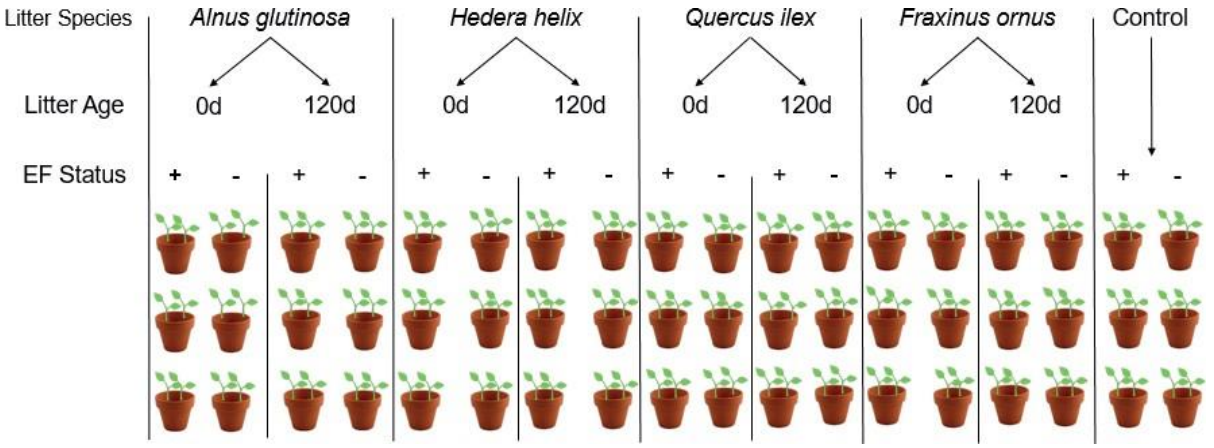

Fig. 1. Schematic view of the experimental design: 3 replicates were made of each endophytic status, 4 litter species were tested (*Alnus glutinosa*, *Hedera helix*, *Quercus ilex* and *Fraxinus ornus*) at two different ages (0 d as undecomposed litter and after 120 days of decomposition); thus, 8 types of experimental units were obtained. + refers to the presence of fungal endophytes and – refers to the fungicide treated seeds and therefore their absence. The total number of the pots is 54 including the 6 of the control without litter. This experimental design applies to *Trifolium repens* (shown in the picture) and *Triticum durum*; therefore, 108 pots were used.

To obtain EF-, the seeds (purchased from commercial secured sources without any previous treatments) were treated with the fungicide Propiconazole (TILT ® 25 EC; 0.25 ml per 100 g of seeds, with a dilution of 1 ml/L) to eliminate the endophyte. Seeds were dipped for 30 min in the fungicide solution and, thereafter, washed three time in distilled water to eliminate treatment residues. Subsequently, seeds were dried in microbiological hood with air laminar flux for 20 min. Fungicide-treated and untreated seeds were cultivated in adjacent 1 m^2 trays in the laboratory for a pre-growth phase to avoid the litter shock due to seed direct contact with litter, and the seedlings were gently pulled to preserve the roots and put back into the experiment corresponding pots as EF- and EF+ seedlings, respectively. The isolation test on 2% malt extract (Difco) agar medium, as reported by Hata et al (1998), confirmed the absence of endophytic fungi in the treated seeds. The isolation test was assessed as follow: after removing the basal sheath, seeds were dipped in 70% ethanol for 1 min to wet the surface, surface sterilized for 15 min in a solution of 15% hydrogen peroxide, dipped again for 1 min in 70% ethanol, and then rinsed in sterilized distilled water. From the surface-sterilized seeds, several segments approximately 5 mm long were aseptically cut off with a sterile scalpel. The segments were then placed on 2% malt-extract agar medium in a 9 cm diameter plate, incubated at 20°C for 21 days, and then checked for presence of fungal colonies.

The experimental pots were filled with soil (23.48% sand, 40.14% silt, 36.38% clay, total C: 10.2 $g.Kg^{-1}$, total N: 3.10 $g.Kg^{-1}$ with an electrical conductivity of 0.29 $dS.m^{-1}$) previously collected (upper 10 cm) from a farm located in the Campania Region, southern Italy (E:14°18′, N:40°51′; 4 m a.s.l.). The soil was sterilized before the start of the experiment by autoclaving at 1 atm pressure, 120°C, for 1 h, three times with 24 h interval. Pots were kept in a greenhouse and were periodically watered to field capacity. To avoid contamination among different pots through leaching or splashing when watering, each pot was located inside an individual plastic container. After 90 days, plants were harvested, the shoots were clipped at soil surface and roots were washed from the soil. Shoots and roots were dried at 70°C in a ventilated chamber for 3 days, and their dry weight recorded.

2.3.5 Statistical analysis

Data obtained from the pot experiments were evaluated using a factorial ANOVA tests to determine the main and interactive effect of the fixed factors: target plants, leaf litter species, decomposition age and endophytic fungi status. Three-way ANOVA was done for each target species testing the effect of litter species, litter age and presence of endophytic fungi. Duncan's pairwise comparisons test was used to compare individual means. Levels of significant differences were assessed at $p<0,05$. All analyses were performed with STATISTICA 13.3 software.

2.4 Results

2.4.1 Litter chemical traits

Leaf litter chemical traits displayed a broad variability with C/N and lignin/N ratios being higher for *Q. ilex* and lower for *H. helix* and *A. glutinosa* (Table 1). Expectedly, C/N ratio decreased with litter age for *F. ornus* and *Q. ilex* while for *A. glutinosa* and *H. helix* it remained almost unchanged. However, lignin/N ratio consistently increased along the decomposition in all litter species with a significant increase for the *H. helix* and *Q. ilex*. Lignin concentration changed during decomposition and with litter species, generally increasing with litter age. Litter N content, on the other hand, has barely varied during decomposition with a slight decrease in the case of *A. glutinosa* and *H. helix*, and a slight increase for *F. ornus* and *Q. ilex*. Differently, cellulose content increased with litter age for *A. glutinosa* while it has highly decreased in the case of *H. helix* and *Q. ilex*. For *F. ornus*, cellulose content showed no changes with litter age.

Table 1. Litter chemical traits of the four tested plant species at two decomposition stages assessed by elemental and proximate analyses. Different letters indicate significantly different groups (Duncan test, $p < 0.05$).

Litter	Age	Labile C (%)	Cellulose (%)	Lignin (%)	N (%)	C/N	Lignin/N
A. glutinosa	0	79.38a	8.46a	10.55a	2.07a	21.73a	5.09a
	120	46.64b	26.43b	18.71b	2.02a	22.27a	9.28a
H. helix	0	70.46a	23.36a	5.77a	2.00a	22.5a	2.89a
	120	60.52a	10.91b	26.49b	1.82a	24.72a	14.55b
F. ornus	0	74.08a	15.54a	10.02a	1.65a	27.27a	6.09a
	120	62.12a	15.35a	20.13b	2.05a	21.95b	9.82a
Q. ilex	0	58.74a	22.74a	18.39a	1.39a	32.37a	13.23a
	120	41.13a	7.79b	50.13b	2.22a	20.27b	22.58b

2.4.2 Plant response to litter amendment and presence of endophytic fungi

The total biomass of both *T. durum* and *T. repens* was affected significantly by leaf litter species, decomposition age, and seed-associated endophytic fungi (Tables 2 and 3). Without endophytic fungi, litter from different species showed variable effects before and after decomposition processes. In general, a significant inhibitory effect on total biomass was evident for undecomposed leaf material of forbs (*H. helix*) and woody species (*Q. ilex* and *F. ornus*) (Fig. 2). However, the undecomposed litter of the nitrogen fixing *A. glutinosa* showed no inhibitory effects on *T. repens* (Fig. 2). Nevertheless, the decomposed litter has shown less inhibition, no inhibition or in some cases, a growth promoting effect. For example, the litter of *A. glutinosa*, *H. helix* and *Q. ilex* showed a growth promoting effect on *T. repens* after the decomposition process. Statistical analyses showed that the interactive effect of leaf litter species and decomposition age affected significantly total biomass of *T. repens* but not of *T. durum* (Tables 2 and 3). Thus, for *T. durum*, the fresh litter encloses more inhibitory effect than the decomposed one for all tested litter species. While for *T. repens*, the same pattern is not confirmed, because both fresh and decomposed litter can exhibit a high inhibition effect on plant total biomass.

Table 2. Summary of the Factorial ANOVA testing for main and interactive effects of EF on *Triticum durum* total biomass in the presence of different litter species at different ages of decomposition. *: The mean difference is significant at the 0.05 level.

Categorical predictor	Sum squares	Degree freedom	Mean Squares	F-value	p-value
Litter species	2.28	3	0.76	7.9	0.000*
Decomposition age	31.10	1	31.10	323.1	0.000*
EF status	1.30	1	1.30	13.5	0.001*
Litter species * Decomposition age	0.28	3	0.09	1.0	0.415
Litter species * EF status	0.19	3	0.06	0.7	0.580
Decomposition age * EF status	0,62	1	0.62	6.4	0.017*
Litter species * Decomposition age * EF status	0.09	3	0.03	0.3	0.813

Table 3. Summary of the Factorial ANOVA testing for main and interactive effects of EF on *Trifolium repens* total biomass in the presence of different litter species at different ages of decomposition. *: The mean difference is significant at the 0.05 level.

Categorical predictor	Sum squares	Degree freedom	Mean Squares	F-value	p-value
Litter species	114.9	3	38.31	252.3	0.000*
Decomposition age	39.6	1	39.62	260.9	0.000*
EF status	5.0	1	4.96	32.7	0.000*
Litter species * Decomposition age	10.8	3	3.60	23.7	0.000*
Litter species * EF status	2.3	3	0.78	5.1	0.005*
Decomposition age * EF status	1.3	1	1.28	8.4	0.007*
Litter species * Decomposition age * EF status	6.1	3	2.03	13.4	0.000*

Endophyte free plants showed an improvement of the seedlings capacity to tolerate fresh undecomposed litter inhibitory effect when compared to untreated plants (Fig. 2). This applies to all undecomposed tested litter species; the total biomass was higher without endophytic fungi and the seedlings showed more sensitivity to litter with their presence. However, in the control, seed associated endophytic fungi exhibited a beneficial effect by increasing the total biomass (Fig. 2). After 120 days of decomposition, the inhibitory effect of litter decreased in all litter species tested, although the effect was not completely disappeared as in the case of *T. repens* in presence of *F. ornus* and *H. helix* litter. It has been observed, in this case, that as long as the litter stress remains, the presence of endophytic fungi enhances the disadvantageous effect of litter, while in the absence of litter inhibitory effect the presence of fungi increase the total

biomass (Fig. 2). However, for *H. helix* and *Q. ilex* decomposed litter, the absence of fungal endophytes promoted the total biomass of *T. repens*. Meanwhile in *T. durum*, results showed that decomposed litter demonstrated no effect on the plant total biomass, both in the absence and the presence of seed-associated endophytic fungi.

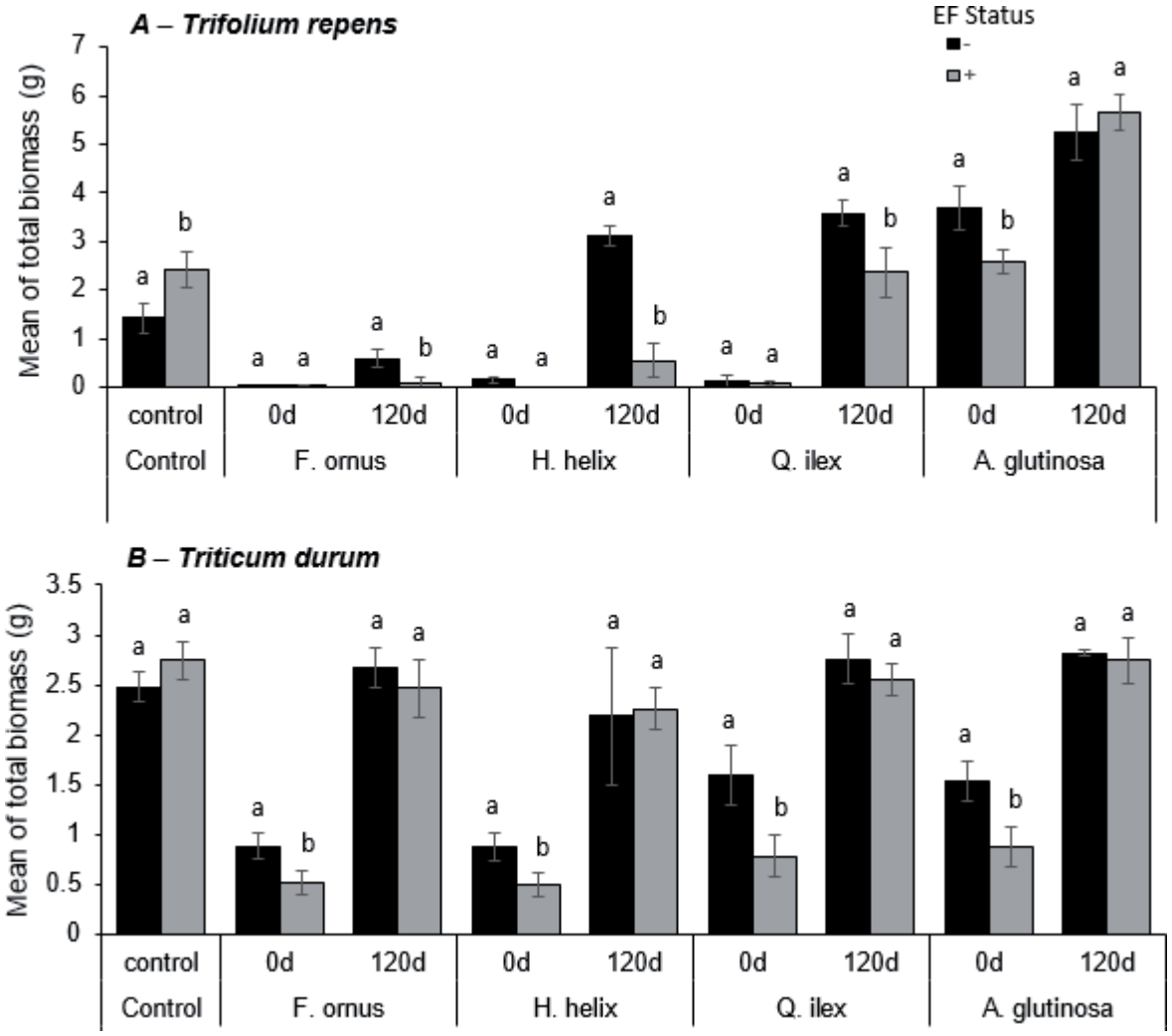

Fig. 2. Mean of total biomass (g.plant^{-1}) of *Trifolium repens* (A) and *Triticum durum* (B) in the presence of four litter species: *A. glutinosa*, *F. ornus*, *H. helix* and *Q. ilex*, at two ages: before (0 d) and after 120 days of laboratory decomposition (120 d), in the presence or absence of seed-associated endophytic fungi (- and +, respectively). Data are represented as means SD (n = 3). Different letters indicate significant differences among treatments for each plot (Duncan test, $p < 0.05$).

2.5 Discussion

Our results confirmed that undecomposed litter had broad inhibitory effects on plant growth (Bonanomi et al. 2017). The phytotoxicity of leaf litter seems to be a general phenomenon, not

limited to some allelopathic species, since all the fresh undecomposed litter species tested produce significant inhibitory effects on the total biomass, with exception of *A. glutinosa* fresh litter on *T. repens*. Moreover, the present study indicates that the inhibitory effect of litter is significantly influenced by both litter species and age of decomposition (Chou et al. 1976, Bonanomi et al. 2006, Zhang et al. 2015).

In contrast to allelochemicals directly extracted from living organs or plant tissues, the activity of organic compounds released from decomposing leaf litter is highly affected by the soil, thus their concentration, composition, structure, and effect might be remarkably different (Yenish et al. 1995, Blum et al. 1998, Walker et al. 2003). The necessary conditions for the appearance of inhibitory effects occur when allelochemicals reach the receiver plant in their active structure and at an effective concentration; thereby undecomposed and decomposed litter often displays different level of inhibition (Zhang et al. 2015). In this study, undecomposed leaf litter of all species exhibited a strong inhibition of the growth. The intensity of phytotoxicity was high in the case of *H. helix* and *F. ornus*, followed by *Q. ilex* while *A. glutinosa* proved to be the least toxic. Bonanomi et al (2011) have demonstrated the existence of a weak but positive correlation between N release and root growth inhibition, excluding that the inhibition exhibited by *H. helix* and *F. ornus* is due to N immobilization, given the large N release by these litter materials during decay. However, after 120 d of decomposition, the phytotoxicity is known to decrease significantly, with a corresponding increase of the seedlings total biomass. Indeed, in the case of *A. glutinosa*, the decomposed leaf litter has even demonstrated a biomass increasing effect instead of phytotoxicity for *T. repens*. Correspondingly, Zhang et al (2015) have demonstrated that an extract of decomposed leaf litter of *Eucommia ulmoides* has accelerated the growth of roots, whilst an extract of undecomposed litter had resulted in significant inhibition. Accordingly, Inderjit (2005) stated that soil microbes were able to break down litter allelochemicals into inactive substances. Moreover, litter after decomposition is considered a source of different nutrients. It has been proven that these nutrients lessen the allelopathy and the associated production of stable organic matter helps in the absorption of allelochemical compounds such as caffeic acids, ferulic acid and salicylic acid, which soften their phytotoxicity (Loffredo et al. 2005). Therefore, all of these interactions can antagonize the negative effects of allelochemicals (Zhang et al. 2015). On the other hand, the inhibition caused by allelochemicals may be increased by soil biochemical conditions. For instance, Pollock et al (2009) reported that when catechins are combined with metal ions, their allelopathic inhibitory effect is strongly

accelerated. These results indicate that under the biochemical and microbial conditions of soil, allelochemical compounds that inhibit the growth can diffuse, decompose or accumulate in the soil and their structure or activities may be altered.

Interestingly and unexpectedly, our study revealed that in the presence of litter stress, seed-associated endophytic fungi have strengthened the inhibitory effect since the total biomass was significantly reduced in their presence. On the contrary, in the control as well after the disappearance of the inhibitory effect because of decomposition, endophytic fungi increased total biomass. This could mean that seed-associated fungal endophytes exhibit their negative effect in the presence of litter allelopathy regardless of the host plant. Omacini et al (2004) found, by measuring litter decomposition rate, that litter decomposition of *Lolium multiflorum* was 17% slower with the *Neotyphodium* endophyte-using microcosms placed outdoors and by nearly 8% under field conditions. However, in the case of *T. repens*, the absence of fungal endophytes in presence of *Q. ilex* decomposed litter has improved the total biomass. This could result from the presence of litter itself rather than its phytotoxicity given that this litter species displayed large difference compared to others in terms of cellulose and lignin contents.

The findings of this study have to be seen in light of a limitation regarding the molecular identification of the naturally seed-associated fungal endophytes of the two focal plants. However, the main goal of the study is to evaluate primarily the general effect of endophytic fungi on the plant response to litter stress. Previous studies have reported that *T. repens* is commonly colonized mostly by dark septate endophytes (Li et al. 2005, Sieber and Grünig 2013), while *T. durum* is mostly colonized by fungal endophytes belonging to the Ascomycetes phylum (Sadrati et al. 2013). Moreover, we lack an integrated understanding of the mechanisms by which endophytic fungi respond differently in the presence and the absence of different litter species. Previous works have suggested various explanations. First, it could be that these fungi affect the plant-associated microbiota in the soil directly by secreting toxic alkaloids as a response to allelopathic stress. For example, some studies have reported negative interactions between fungal endophytes and secondary decomposer fungal saprotrophs (Dowson et al. 1988, Fukasawa et al. 2009). Moreover, the reason could be the control of root exudate by endophytic fungi, these latter may alter some metabolic pathways of their host plant, resulting in several changes in litter components and in an increase in toxic substances (Schmidt et al. 1982, Lyons et al. 1990, Purahong and Hyde 2011). Furthermore, endophytic fungi live inside the plant tissues in a continuum lifestyle, ranging from mutualistic to pathogenic depending on the outside conditions (Saikkonen et al. 1998). Therefore, these results may be explained by

the modulation of the activity of associated endophytic fungi that shift from mutualistic to opportunistic pathogenic under the allelopathy stress, which has reinforced the inhibitory effect of allelochemicals. It will be very interesting to look at interactions between the endophytic microbiome and the occurrence of the inhibitory effect due to self-DNA (Mazzoleni et al. 2015). Also in the case of autotoxicity, the dynamics of decomposition could have different outcomes mediated by plant-microbes interactions so far neglected.

The negative effect of seed-associated endophytic fungi on the total biomass in the presence of litter demonstrates that these microbes have an indirect effect on the plant-litter interaction. Our data suggest that seed-associated endophytic fungi may have an important role in the plant community dynamics by affecting plant-plant interaction and possibly modulating species coexistence. Along this line, a recent study demonstrated that *Centaurea stoebe* L. cannot only expand, but can perform better in new ranges indirectly by escaping their associated endophytes (Geisen et al. 2017).

2.6 Conclusion

In this study, we found that, aside from the important role of leaf litter decomposition in biogeochemical cycles, litter also has a strong effect on plant-plant interaction that depend on presence of endophytic fungi. In detail, seed-associated endophytic fungi demonstrated to enhance the inhibitory effect of litter and could affect plant coexistence. Further studies are required to understand the exact mechanism by which fungal endophyte exhibit their role in affecting plant-litter interaction and in structuring plant communities. Special attention should be given as well to the effect of these microbes on the plant growth and tolerance in the presence of conspecific litter.

3 Chapter 3: Contrasting effects of *Rhizophagus irregularis* versus bacterial and fungal seed endophytes on *Trifolium repens* plant-soil feedback

3.1 Abstract

Interactions between plants and soil affect plant–plant interactions and community composition by modifying soil conditions during plant-soil feedback in which associated microbes have the most important role. Both arbuscular mycorrhizal fungi (AMF) and microbial seed endophytes have been demonstrated to influence, directly or indirectly, biotic or abiotic soil properties and thus to affect subsequent plant growth and community structure. However, little is known about how plant endophyte communities, individually or in interaction with AMF, affect plant-soil feedback processes. Here, we investigated through a manipulative experiment the behavior of endophyte-free and endophyte-associated *Trifolium repens* plants grown in soils previously conditioned by conspecific endophyte-free and endophyte-associated plants, inoculated or not by *Rhizophagus intraradices*. Furthermore, we identified microbial endophytes directly from the inner tissues of seeds by high-throughput sequencing, to compare seed fungal and bacterial endophyte composition. Results demonstrated that the outcome of simultaneous occurrence of seed endophytes and AMF on plant behavior depended on the endophytic status, i.e. either presence or absence of seed microbial endophytes, match between the conditioning and response phases. Seed fungal endophytes generated strong conspecific negative feedback, while seed bacterial endophytes shifted the feedback from negative to positive. Moreover, the simultaneous occurrence of both seed endophytes with AMF could either generate or intensify negative plant-soil feedback effects. Our results show that seed and root symbionts can play a significant role influencing conspecific plant-soil feedback.

Keywords: arbuscular mycorrhizal fungi, soil-borne pathogens, *Rhizophagus intraradices*, fungal endophytes, bacterial endophytes

3.2 Introduction

It is known that soil microorganisms may differ in their relative abundance within the rhizosphere in relation to soil types and plant species (Bever et al. 2010). This soil microbiome can negatively or positively influence plant performance and competitiveness in a way that may directly or indirectly shift plant community composition, under a sum of interactions called 'plant-soil feedback processes' (Van der Putten et al. 1993; Bever et al. 1997; Van der Putten & Peters 1997; Bartelt-Ryser et al. 2005). The direct influence of soil microbes is shown through unequivocal interactions between plant roots and pathogens (Bruehl 1987), mutualists (Smith and Read 1997), and parasites, which exhibit a spectrum of effects on their host plant, ranging from benefit to damage, such as mycorrhizal fungi when their net cost of the symbiosis exceeds the benefits (Johnson et al. 1997). Indirect impact of soil microbes relies upon their interactions with decomposing and mineralizing soil organisms (Bardgett and Shine 1999, Wardle et al. 2003, Hättenschwiler et al. 2005) or upon belowground trophic cascades (Strong et al. 1996). Plant-soil feedback is defined as the interaction between plants and their biotic and abiotic soil environment, which subsequently influences the performance of the same or other plant species in following generations (Bever et al. 1997; Ehrenfeld et al. 2005). In addition, plants may influence the performance of conspecifics and heterospecifics by affecting the chemical composition of the organic substrate and inducing auto-toxicity by extracellular DNA (Mazzoleni et al. 2015). Both the magnitude and the direction of plant-soil feedback represent major factors driving plant community succession, which have been predicted theoretically to switch in parallel with environmental changes (Reynolds et al. 2003). Typically, plant-soil feedbacks are investigated using a two-phase experiment (Bonanomi and Mazzoleni, 2005; Kulmatiski et al., 2008; Brinkman et al., 2010).

Plant performance and disease resilience can be improved markedly by different microbial communities such as bacteria and fungi. The major classes of ubiquitous microbial symbionts associated with plants in terrestrial ecosystems are arbuscular mycorrhizal fungi (AMF) and microbial endophytes, in particular, fungal and bacterial endophytes. More than 80% of terrestrial plant roots are colonized by AMF (Smith and Read 2008). These offer the host plant several benefits in exchange for photosynthesis products such as hexoses and lipids (Keymer et al. 2017). They increase the volume of exploited soil and hence increase mineral nutrient uptake (Smith and Read 1997), they improve tolerance of water stress (Porcel and Ruiz- Lozano 2004) and soil salinity (Daei et al. 2009), and they are involved in pathogen suppression (Cordier et al. 1998) and toxic metals resistance (Turnau et al. 2006). Several

recent studies suggest that AMF symbiosis moderates the competitiveness, community structure, and the diversity of plant species (Lin et al. 2015; Bennett et al. 2017; Teste and Dickie, 2017) which consequently contributes to the success/failure of plant invasion (Gerz et al. 2018). Contemporary studies based on molecular identification of fungal taxa supported the hypothesis that AMF abundance, composition and traits may affect the survival, establishment and invasion of host plants in new habitats (Yang et al. 2014; Neuenkamp et al. 2018).

During the past decades, microbial communities of the rhizosphere and the phyllosphere have received extensive attention regarding their roles and development. However, microbiota inhabiting other niches, such as seeds, have been poorly studied and understood. Seeds form an important habitat for microbes, sustaining a diverse array of both harmful and beneficial ones (Nelson, 2004). Recently, research has revealed that seed endophytes have the ability to promote seed germination and enhance plant growth during both abiotic and biotic stress (Truyens et al., 2015). Therefore, it is reasonable to hypothesize that seed-associated endophytes play a more important role in modulating their host plant than previously thought.

Microbial endophytes are bacteria and fungi that asymptomatically inhabit host plant tissue for a portion of their life cycle (Stone et al. 2000). In some cases, they exhibit mutualistic interactions by protecting the host against biotic and abiotic stresses and pests in exchange for their own protection and nutrition (Hardoim et al. 2015). The evolution of endophytes has been occurring along with that of plants, thus they have developed different functional and survival strategies in close relationship with their host plants (Krings et al. 2007; Yu et al. 2010; Selim et al. 2012; Goyal et al. 2017). Evidence suggests that the presence of microbial endophytes may not only affect plant growth, fitness, and diversity but also population dynamics, plant competitiveness, and ecosystem functioning (Saikkonen et al. 1998; Hardoim et al. 2015). Impacts upon the competitive ability of their host plant by endophytes can be due to the direct impact of their products which induce many changes for the host such as shoot and root growth adjustment (Takai et al. 2010; Craig et al., 2011), physiological responses to abiotic stresses (Saari & Faeth, 2012; Wu et al., 2016), resistance to herbivory (Clay et al. 2005), or production of allelopathic substances (Rudgers & Clay, 2007; Bao et al., 2015). Endophytes' interactions with other symbionts, such as AMF and rhizobacteria, can have indirect effects on plant growth and fitness as well (Omacini et al. 2006).

Endophytes and AMF are distinguished by different traits and lifestyles: endophytes do not form any obvious physical interaction structures within plants while mycorrhizal fungi do. Moreover, according to Brundrett (2002), the development of fungal endophytes in contrast to mycorrhizal fungi is not synchronized with the development of their hosts. Thus, fungal

endophytes may complete their lifecycle outside the host organism and so are able to grow on artificial media (Petrini, 1996). Furthermore, mycorrhizal fungi are restricted to the roots of plants, while endophytes also can colonize aboveground organs such as leaves, stems, flowers, and seeds. Despite these differences, endophytes and AMF both can establish strong mutualistic associations with the host plant and receive proper habitat, protection, and organic carbon supply (Binet et al., 2013; Mack & Rudgers, 2008). Several investigations have reported interactions of host plants with either of these types of microbes (Klironomos 2002; Bever 2002; Matthews and Clay 2001; Cripps et al. 2013), but studies of the simultaneous interaction between endophytes and AMF on the same host plant are uncommon (Liu et al., 2011). To date, most studies (Chu-Chou et al., 1992; Müller, 2003; Mack & Rudgers, 2008; Liu et al., 2011; Larimer et al. 2012) have primarily investigated the effect of the interactions between endophytes and AMF on plant performance, largely ignoring investigation of possible other interactions between the symbionts and the soil environment.

Simultaneous association with both types of symbionts can be positive, negative or neutral for host growth depending upon the plant species, AMF and endophyte genotypes, and depending on the environmental context (Omacini et al. 2006; Mack and Rudgers 2008; Zhou et al. 2016; García-Parisi & Omacini, 2017). Simultaneous colonization by both endophytes and AMF on a common host plant may affect not only the plant itself but additionally may alter the next generation of the host plant via changes in the soil through plant-soil feedback processes. A recent study by García-Parisi & Omacini (2017) showed that AMF could shift feedback effects between fungal endophyte-colonized and fungal endophyte-absent plants from negative to positive. However, whether the interaction between seed bacterial endophytes and AMF affect the next generation of plants never has been studied. Indeed, fungal seed endophytes have been reported to induce negative plant-soil feedback (Matthews and Clay 2001), and their association with AMF has been described as antagonistic, negatively affecting host growth and survival (Larimer et al. 2012). Therefore, we hypothesized that such an association would increase the intensity of plant-soil negative feedback due to its reported antagonism on the host. Additionally, our work investigated the simultaneous effects of bacterial seed endophytes in association with an arbuscular mycorrhizal fungus on plant-soil feedback processes. We hypothesized that seed bacterial endophytes associations with AMF would generate a positive plant-soil feedback, because each of these microbes has been shown to have multiple benefits for the host plant (Smith and Read 1997; Cordier et al. 1998; Porcel and Ruiz- Lozano 2004; Turnau et al. 2006; Daei et al. 2009; Hardoim et al. 2015).

3.3 Material and Methods

The experiment was carried out in a greenhouse between February and August 2018. The experiment was divided into two phases: the conditioning and the response phase. The target species used in this experiment, *T. repens*, was chosen for its short life span, being an agricultural annual, and because it is a nitrogen fixing species. Certain endophytic bacteria are among N-fixing bacteria, so using an N-fixing plant species would help to make clear their effect. The seeds used in this experiment were collected after growing plants derived from commercially purchased seeds with no previous treatment (De Corato sementi^{R}), for three successive reproductive cycles.

3.3.1 Soil conditioning phase

In this phase, plants were grown in the soil long enough to condition it and alter the local soil biotic and abiotic conditions (Ehrenfeld et al., 2005; Van der Putten et al., 2013).

Here, pots (19 cm top diameter * 17 cm height * 14 cm base diameter) were filled with sterile soil (23.48% sand, 40.14% silt, 36.38% clay, total C: 10.2 g. Kg^{-1}, total N: 3.10 g. Kg^{-1}, C/N ratio: 3.29, P_2O_5: 241.74 mg kg^{-1}, with an electrical conductivity of 0.29 $dS.m^{-1}$) collected (upper 10 cm) from a farm located in the Campania Region, southern Italy (E:14°18′, N:40°51′; 4 m a.s.l). The soil was sterilized before the start of the experiment by autoclaving at 1 atm pressure, 120 °C, for 1 h, three times with 24 h intervals.

To obtain endophytic fungi-free seeds, seeds were treated with Propiconazole fungicide (TILT ® 25 EC; 0.25 ml per 100 g seeds, with a dilution of 1 ml. L^{-1}) to eliminate the endophytes. Seeds were dipped for 30 min in the fungicide solution and, thereafter, washed three times in distilled water to eliminate treatment residues. Subsequently, seeds were dried in microbiological hood with air laminar flow for 20 min. An isolation test on 2% malt extract (Difco) agar medium, as implemented by Hata et al (1998), confirmed the absence of endophytic fungi in the treated seeds. The isolation test was assessed as follows: after removing the basal sheath, seeds were dipped in 70% ethanol for 1 min to wet the surface, surface sterilized for 15 min in a solution of 15% hydrogen peroxide, dipped again for 1 min in 70% ethanol, and then rinsed in sterilized distilled water. From the surface-sterilized seeds, several segments approximately 5 mm long were aseptically cut with a sterile scalpel. The segments were placed on 2% malt extract agar in a 9 cm diameter plate incubated at 20°C for 21 days and then were checked for presence of fungal colonies. After the incubation period, no fungi culture was obtained, while many cultures were obtained from the untreated seeds. Moreover,

a germination test on Petri dishes demonstrated the absence of an inhibitory effect of the fungicide on the growth of seedlings compared to controls.

To obtain seeds without endophytic bacteria, seeds were soaked for 20 minutes in an antibiotic solution containing a combination of chloramphenicol (500 µg.ml^{-1}) and tetracycline (12 µg. ml^{-1}) (Puente et al. 2009). An isolation test on LB agar medium, as implemented by Herrera et al. (2016), confirmed that no bacteria culture was obtained from the treated seeds in contrast to untreated seeds where evident cultures were obtained, and a germination test on Petri dishes demonstrated the absence of an inhibitory effect of the antibiotic combination on the growth of seedlings compared to controls without treatment.

The arbuscular mycorrhizal (AM) fungus inoculum consisted of *Rhizophagus irregularis* spores (commercial culture: Mykos XtremeR, 300-propagules. g-1). Plants of *T. repens* were grown in 36 pots (ten plants per pot). 12 pots were sown with untreated seeds i.e. control, 12 with EB- i.e. bacteria-free seeds, and 12 with EF- i.e. fungi-free seeds. This conditioning prepared for two subsequent independent response experiments involving bacterial or fungal seed endophytes. Moreover, 30 g of AM fungus inoculum was added to half the pots of each treatment (6 pots of each 12). Thereby, six treatments each replicated six times were obtained. On 15th June 2018 (after 3 months), shoot tissues were cut and the soil was sieved to be used in the response phase. For each seeds treatment, one type of the endophytes is confirmed to be absent while the other type is still present (bacterial endophytes are still present in EF- seeds and fungal endophytes are still present in EB- seeds). However, because the comparison is done against the control in which both seed endophytes are present, the observed effect is assumed to be resulted from each seed endophytes absence.

3.3.2 Response phase

During the response phase, nine small pots (17 cm top diameter * 15 cm height * 12,3 cm base diameter) were filled from the six large pots of each conditioned treatment. 5 seeds were sown in each response pot, with the same seed treatments as those applied for the conditioning phase. Accordingly, and during this phase, we obtained response plants growing in soils conditioned by plants with their same endophytic status (i.e. EB+/EF+ plants growing in soils conditioned by EB+/EF+ control plants, and EB- or EF- plants in soils conditioned by EB- or EF- plants), and additionally, response plants growing in soils conditioned by plants with different endophyte symbiotic status from their own (i.e. control plants growing in soils conditioned by EB- or EF- plants, and EB- or EF- plants in soils conditioned by control plants) (Fig 1).

The response phase was carried out for three months (from mid-June to August 2018) in a greenhouse. Each pot was located inside an individual pot saucer to avoid contamination among plants through leaching or splashing when watering. Pots were watered to field capacity, when necessary, by adding distilled water to the individual pots.

After three months, and before of the end of the plant cycle, response plants were harvested. Shoots were clipped, roots were washed, and rhizobia nodules were counted. The origin of the nodules is thought to be from the fact that rhizobia are a common greenhouse contaminant or from the previous cultivation of legumes in the field soil and rhizobia that survived autoclaving by forming heat-resistant spores. All shoots and roots were dried at 70 °C for 72 hours, and their dry weight recorded. Moreover, the roots were analyzed for the presence or absence of *R. irregularis* colonization by microscopic observation. However, no quantification of the AM fungus was conducted.

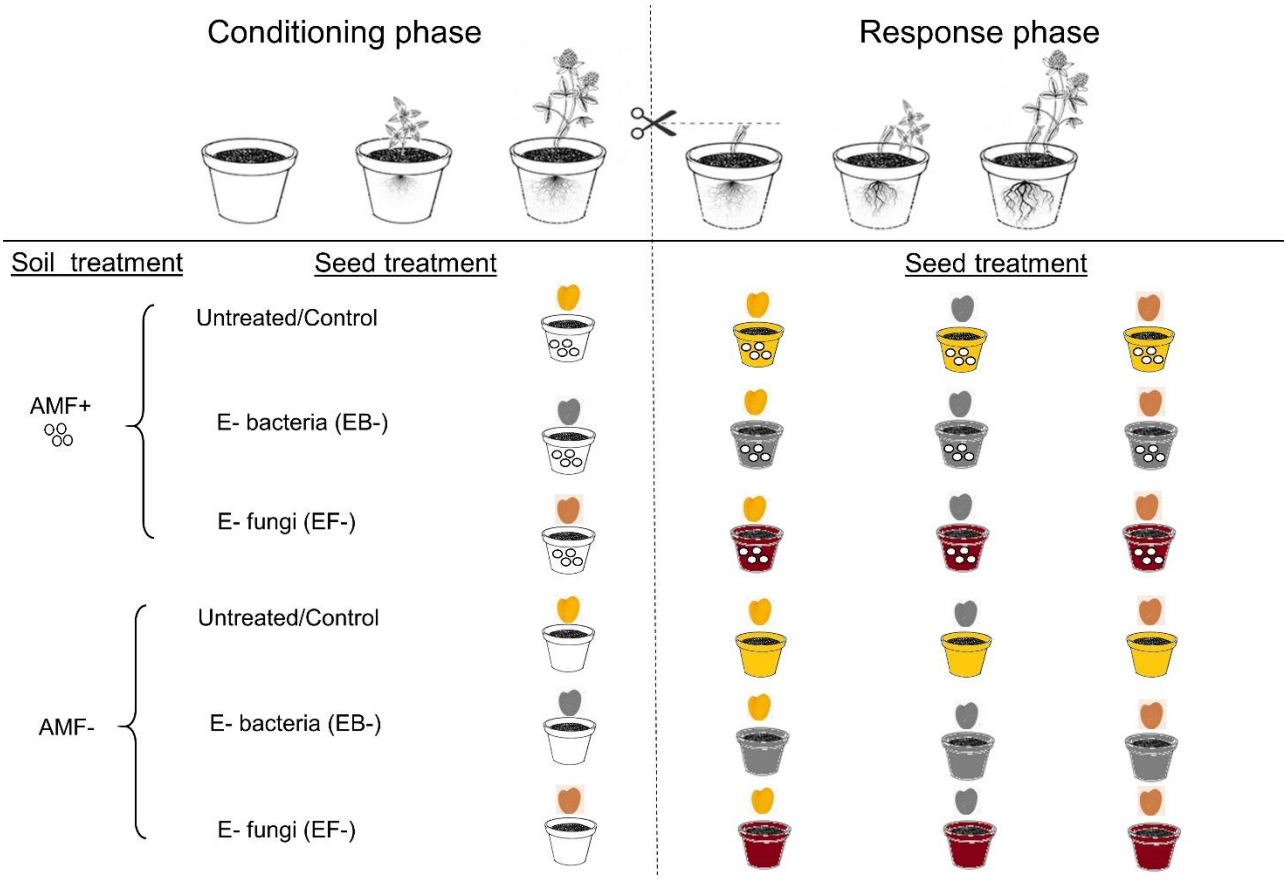

Fig 1. Representative schema of the two phases of the experiment design. The initial phase was conditioning of the soil by endophyte and endophyte-free seeds with or without inoculation of the arbuscular mycorrhizal fungus (AMF+ and AMF-, respectively). The pots in the subsequent response phase are shown with the color of the seed treatment during the conditioning phase, thus, this phase consists of the growth of the same seed types, i.e., Untreated, EB- and EF- in each type of the

conditioned soil, resulting in E- and E+ plants growing in soil conditioned by plants with the same bacterial or fungal seed endophytic status or soil conditioned by plants with different bacterial or fungal seed endophytic status, along with or without the AM fungus. The total number of response phase pots is 54 pots (3 response seed treatments * 3 types of conditioned soils * 2 AMF status * 3 replicates).

3.3.3 Microbiological analyses

The microbiome of seed microbial endophyte communities was analyzed by Illumina high-throughput sequencing. The analyzed seeds were those used for the conditioning phase controls. A PowerSoil DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, CA) was used to isolate the DNA from 2 g of surface-sterilized seeds for each of three replicates. Seed surface sterilization prior to DNA isolation was performed by serial washes using 70% ethanol for 5 min, 1.5% sodium hypochlorite for 15 min and several rinses with distilled water over a 20 min period (López-López et al. 2010). The surface sterilized seeds were crushed in a sterile mortar and pestle and suspended in sterile saline solution (0.85% NaCl) for 2 h at 28°C under shaking. Two grams of crushed suspended seeds were then transferred, aseptically, into each of three Eppendorf tubes for DNA extraction.

Bacterial and fungal diversity was assessed by high-throughput sequencing of the amplified V3-V4 regions of the 16S rRNA gene (~460 bp) and ITS1-2 (~300 bp). PCR was carried out with primers S-D-Bact-0341-b- S-17/S-D-Bact0785-a-A-21 (Berni Canani et al., 2017) and BITS1fw/ B58S3- ITS2rev (Bokulich & Mills, 2013) using conditions reported in the original studies. For bacterial primers S-D-Bact-0341-b-S-17 (5’ -CCTACG GGNGGCWGCAG-3’) and S-D-Bact-0785-a-A-21 (5’ -GAC TACHVGGGTATCTAATCC-3’), PCR conditions were: 25 cycles of 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 72°C for 5 min and held at 4°C. For fungal primers BITS1fw (5’-ACCTGCGGARGGATCA-3’) and B58S3-ITS2rev (5’-GAGATCCRTTGYTRAAAGTT-3’) PCR conditions were: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension of 72°C for 5 min. PCR products were purified with the Agencourt AMPure beads (Beckman Coulter, Milan, IT) and quantified using an AF2200 Plate Reader (Eppendorf, Milan, IT). Equimolar pools were obtained prior to further processing, and were pyro-sequenced on an Illumina MiSeq platform, yielding 2× 250 bp, paired-end reads.

3.3.4 Bioinformatics and statistical analyses

Raw reads were filtered and analyzed with the QIIME 1.9.1 software (Caporaso et al. 2010). Reads shorter than 300 bp or 150 bp (for bacteria and fungi, respectively), with more than one primer mismatch and with an average quality score lower than 25, were discarded. OTUs were

picked through a de novo approach and the UCLUST method, and taxonomic assignment was obtained by the RDP Classifier and the Greengenes (McDonald et al. 2012) or the UNITE v.8 database (Quast et al. 2012). Contamination by chloroplasts and Streptophyta, as well as singleton OTUs, were removed and the relative abundances of other taxa were recalculated. To avoid biases due to different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample (77038 for ITS and 115888 for 16S). Raw sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number PRJNA587416.

Statistical significance of biomass data obtained from the experiments was evaluated using three-way, factorial ANOVAs (analysis of variance) in order to determine the main and interactive effects of the fixed factors for prokaryotic and fungal seed endophytes separately: AM fungus status, endophytic status of conditioning phase, and endophytic status of response phase. After ANOVA, means were separated with Duncan's pairwise test. Duncan's post-hoc test was used because of its power, and applicability regardless of whether a significant F resulted from the analysis of variance.

A plant-soil feedback effect was calculated as the ratio between total biomass in conditioned and response soil according (Brinkman et al. 2010). In the present experiment, the feedback effect was calculated as ln (total biomass of response plants growing in soils conditioned by plants with their same endophytic status / total biomass of response plants growing in soils conditioned by plants with different endophytic status). To test significant changes in the feedback effect in the presence of the AM fungus and endophytes, feedback data were analyzed through generalized linear models (GLM) including AM fungus status and endophytic status of the conditioning plants as fixed factors.

Levels of significant differences were assessed at $P<0.05$. All analyses were performed with STATISTICA 13.3 software.

3.4 Results

3.4.1 Seed microbiome

In regards to bacterial richness and diversity of seed-associated endophytes, *Proteobacteria* were the most abundant showing a value greater than 60% of all the bacterial endophytes. In particular, members belonged to the *Enterobacteriaceae* family (>35%), followed by members that belonged to the *Oxalobacteraceae* family (>7%). On the other hand, low relative abundance was observed for the *Actinobacteria* phylum (<8.4%), *Firmicutes* (<7.7%) and

Bacteroidetes (<5.9%). While the rest (<17%) were allocated to the unassigned bacterial community (Fig2, A). With respect to fungal richness and diversity, a high relative abundance was observed for the *Ascomycota* phylum (93.62%), especially *Stemphylium* (28.4%), followed by members belonging to *Alternaria* (14.7%), *Epicoccum* (14.7%) and both the *Nectriaceae* family (10.8%) and the genus *Cladosporium* (9.8%), while other classes represented abundance of more than 10%. However, *Basidiomycota* phylum was observed to be present the least with an extreme low abundance of 1.52%. Only 3.7% of the fungal community was unidentified (Fig 2, B).

3.4.2 *T. repens* performance in the response phase

3.4.2.1 Seed fungal endophytes

The total biomass of *T. repens* was significantly affected by the AM fungus ($F_{1, 16}$ =16.48, P=<0.01), conditioning phase ($F_{3, 16}$ =114.8, P=<0.01), and the interaction between the three factors i.e. AM fungus status, response and conditioning endophytes status ($F_{7, 16}$ =11.04, P=<0.01) (STable 1). In both cases of AMF+ and AMF-, a higher total biomass was recorded when soil was conditioned with EF- compared to EF+ regardless of the response endophytic status. However, a significant increase in the total biomass for AMF+ was recorded when soil was previously conditioned with EF- compared to EF+, especially for EF- response plants. Moreover, the conditioning with EF+ results always with a dramatic low growth regardless of both the AM fungus status and the response endophytic status (Fig. 3, A).

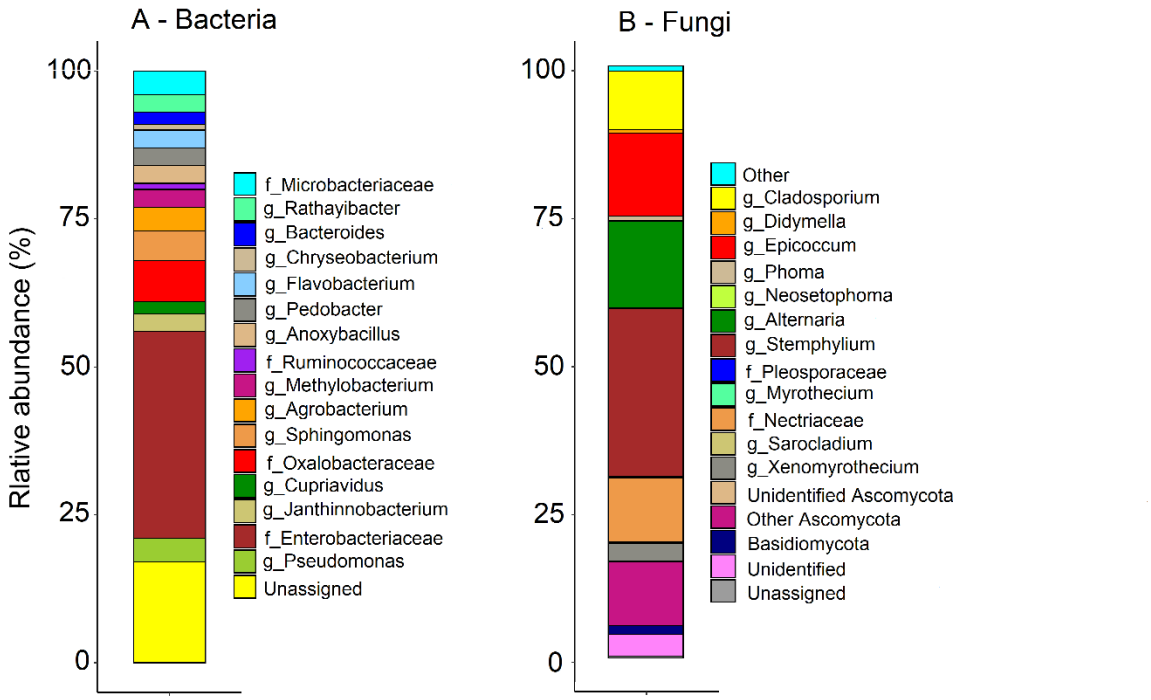

Fig 2. Relative abundance of bacterial **(A)** and fungal **(B)** endophytes inside the tissues of *Trifolium repens* conditioning seeds. g_ in the key refers to genus level and f_ to family level in case of an unidentified genus.

3.4.2.2 Seed bacterial endophytes

The total biomass of *T. repens* was significantly affected by the conditioning phase ($F_{3, 16}$ =7.48, P=0.01), the interaction between the AM fungus and the conditioning seed endophytes status ($F_{5, 16}$ =60.37, P=<0.01), the interaction between AM fungus and the response seed endophytes status ($F_{4, 16}$ =29.83, P=<0.01), and the interaction between the conditioning and the response seed endophytes status ($F_{6, 16}$ =10.48, P=<0.01) (STable 2). For AMF-, higher total biomass was recorded when conditioning with EB+ in comparison to EB-. While for AMF+, soil conditioning with EB+ decreased the total biomass compared to AMF-. For EB+ in the response phase, the total biomass improved in the case of conditioning with AMF+ EB- and decreased in the case of conditioning with AMF- EB+ (Fig. 3, B).

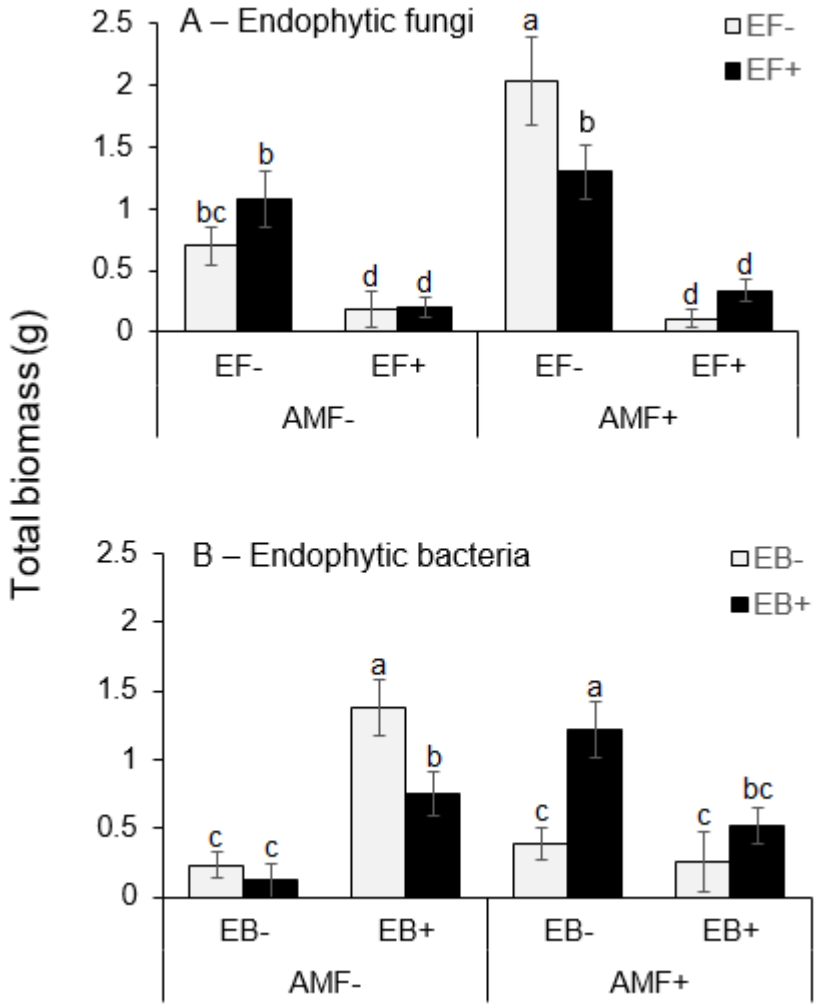

Fig 3. Average of total biomass (g. plant^{-1}) of response endophyte-free and endophyte-associated *Trifolium repens* plants (A: response plants with (EF+) and without (EF-) their seed-associated fungal endophytes; B: response plants with (EB+) and without (EB-) their seed-associated bacterial endophytes) growing in soils conditioned by plants with the same or different endophytic status (indicated on the abscissa) with or without arbuscular mycorrhizal fungus inoculation (AMF+ and AMF-, respectively). Grey and black bar colors represent the status during the response phase. The error bars represent the standard deviation. Bars topped by the same letter do not differ significantly by Duncan test.

3.4.2.3 Rhizobia nodules number

For AMF-, conditioning the soil with EF+ resulted in the absence of rhizobia nodules regardless of the response endophytic status, while conditioning the soil with EF- resulted in the presence of rhizobia, especially for EF+ response plants. However, in the presence of the AM fungus, regardless of the conditioning endophytic status, EF- in the response phase tremendously increased nodules number (Fig. 4, A). On the other hand, when conditioning the soil with EB-, rhizobia nodules were absent for AMF- and were low for AMF+ regardless of the response

endophytic status. However, when conditioning the soil with EB+, rhizobia nodules were present only EB+ in the response phase, with a significant increase when the AM fungus was absent (Fig. 4, B).

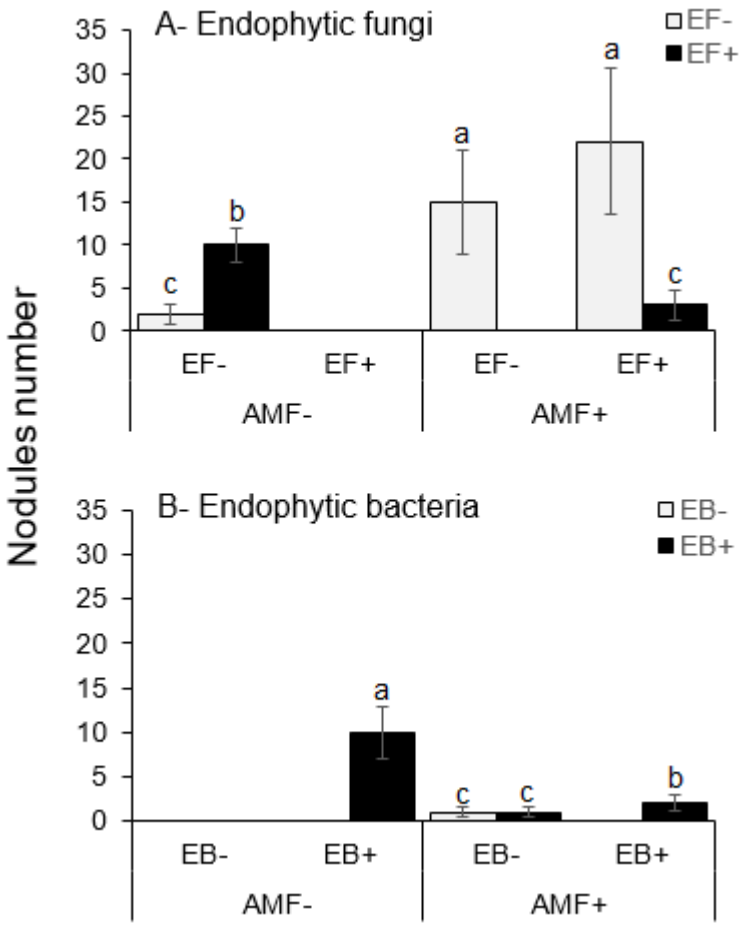

Fig 4. Number of rhizobia nodules of endophyte-free and endophyte-associated *Trifolium repens* response plants (A: fungal endophytes absent (EF-), fungal endophytes present (EF+); B: bacterial endophytes absent (EB-) or bacterial endophytes present (EB+) plants) with or without arbuscular mycorrhizal fungus inoculation (AMF+ and AMF-, respectively). Grey and black bar colors represent the status during the response phase. The error bars represent the standard deviation. Bars topped by the same letter do not differ significantly by Duncan test.

3.4.3 *T. repens* plant-soil feedback

The absence of EF always creates positive plant soil feedback ($F_{1, 30}$ =110.071, P=<0.001) which gets stronger with the simultaneous presence of the AM fungus. However, the presence of EF always generates negative feedback conditioning of the soil regardless of the AM fungus status ($F_{3, 30}$ =3.657, P=0.0654) (Fig. 5, A, STable 3). On the other hand, the absence of EB created a strong negative feedback conditioning ($F_{2, 24}$ =8.836, P=0.007), which shifted to

positive with the presence of the AM fungus. Nevertheless, the presence of EB creates a strong positive feedback, which shifted to negative in the presence of the AM fungus ($F_{3, 24}$ =65.583, P=<0.001) (Fig. 5, B, STable 3).

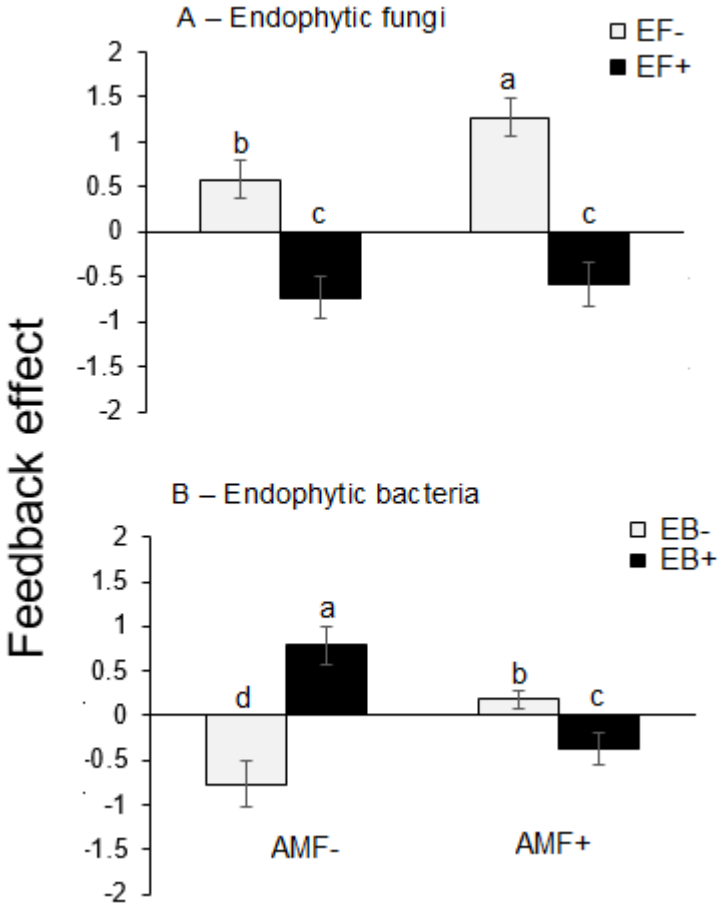

Fig 5. Feedback effect of fungal endophytes (A: fungal endophytes absent (EF-); fungal endophytes present (EF+)) and bacterial endophytes (B: bacterial endophytes absent (EB-); bacterial endophytes present (EB+)) on *Trifolium repens* response-plants. Grey and black bar colors represent the status during the response phase. The error bars represent the standard deviation. Bars topped by the same letter do not differ significantly by Duncan test.

3.5 Discussion

Our investigations have shown that conditioning the soil with the presence of fungal endophytes generated negative feedback, while their absence caused positive feedback regardless of the absence or presence of the AM fungus. On the contrary, conditioning the soil with the absence of bacterial endophytes from seeds developed strong negative feedback conditions in the absence of the AM fungus. Whereas in the presence of bacterial endophytes in the response plant seeds, strong positive feedback was observed in the absence of the AM fungus. For each endophyte type, with endophyte removal from the response plant seeds the

AM fungus showed positive feedback, while its interaction with the seed endophytes resulted in negative feedback. Therefore, AMF may have a key role in modulating conspecific plant-soil feedback processes.

The individual effects of AMF and fungal endophytes, along with their interactions on plant-soil feedback processes have been extensively investigated during the past decade (Hardoim et al. 2015; Zhou et al. 2016; Gerz et al. 2018). AMF have been shown to be capable of affecting, positively or negatively, plant-soil feedback depending on the host plant and the mycorrhizal fungus species (Bever, 2002, 2003; Bennett et al. 2017). On the other hand, the role of fungal endophytes in plant physiology and their possible utilization as growth promotion and stress protection agents recently has been a popular subject. However, previous studies have found that seed fungal endophytes generate a strong conspecific negative feedback (Geisen et al. 2017; Idbella et al. 2019), which aligns with our findings.

For example, Matthews and Clay (2001) found that soil-mediated inhibition of EF+ *Festuca* by previous conditioning with EF+ *Festuca* represents a direct negative feedback that could limit the growth of EF+ *Festuca* relative to other species, thereby reducing or preventing domination by EF+ *Festuca*. However, García-Parisi et al. (2017) demonstrated that conditioning soil with an annual grass and its foliar fungal endophytes impaired the belowground symbiotic potential but did not necessarily result in negative plant-soil feedback on legume performance. Our findings highlight that fungal seed endophytes individually generate negative plant-soil feedback for *T. repens*, while an AM fungus helped in developing positive feedback. However, with the combined presence of AM fungus and fungal seed endophytes, the negative feedback generated by the endophytes remained unchanged. These findings were supported by many previous investigations reporting that the simultaneous association with fungal endophytes and AMF can produce antagonistic effects on host performance depending on AM fungus or endophyte species, and/or nutrient availability (Mueller, 2003; Novas et al. 2005; Mack and Rudgers, 2008; Liu et al. 2011). For instance, Larimer et al. (2012) have stated that fungal endophyte infection differentially altered hyphal colonization by AMF species and the identity of the co-infecting AMF species affected fungal endophyte fitness traits. Nonetheless and distinctively, a recent study by García-Parisi and Omacini (2017) has shown that symbiosis with AMF can be positive for both endophyte-free and endophyte-associated plants depending on matching between the endophytic status of both grass generations. Several possible mechanisms were suggested to explain the antagonistic effects of seed fungal endophytes upon AMF, whether directly through chemical inhibition by the strong production of alkaloids (Spiering et al. 2005; Rasmussen et al. 2007), or indirectly

by altering the nutritional requirements of the host plant, thereby affecting mycorrhizae (Rahman and Saiga, 2005; Blanke et al. 2005), or by competition over resource allocation between symbionts (Brundrett, 2002). Moreover, endophytes have temporal priority relative to AMF because endophytes are present in seeds (vertical transmission) prior to germination, while AMF are transmitted horizontally. Like spatial priority, temporal priority could generate the observed asymmetric and negative relationship between endophytes and AMF (Mack and Rudgers, 2008).

Generally, the literature is centered on the effect of fungal endophytes and their interaction with AMF in plant-soil feedback processes (Mack and Rudgers, 2008; García-Parisi and Omacini, 2017; Geisen et al. 2017; Idbella et al. 2019). Here for the first time, the effect of bacterial seed endophytes was investigated both individually and in association with an AM fungus. We found that these seed endophytes alone succeeded in generating a strong positive feedback while their absence caused a clear shift of the feedback from positive to negative. However, their interaction with the AM fungus unexpectedly generated negative plant-soil feedback. In contrast, previous studies have found that the association between bacterial endophytes and AMF is beneficial for mycorrhization, pathogen control and eventually leads to health and growth improvements for host plants (Offre et al., 2007; Pivato et al., 2009; Sundram et al. 2011). In fact, Hashem et al. 2016 found that endophytic bacteria and AMF that live within the plant tissues of *Acacia gerrardii* are co-ordinately involved in the plant's adaptation to stress tolerance, increased plant growth and nutrient acquisition and improved symbiotic performance of *A. gerrardii*. In addition, endophytic bacteria have shown a synergistic effect upon AMF by increasing their germination and root colonization. Yet, antagonistic effect in the interaction between seed bacterial endophytes and AMF has not been reported in the literature so far. However, similarly to fungal endophytes, competition over resources allocation and temporal priority could explain the negative effect generated by the interaction of seed bacterial endophytes and an AM fungus.

Our results indicated that when the AM fungus was absent and both seed bacterial and fungal endophytes were present, the number of rhizobacteria nodules increased significantly, while in the presence of the AM fungus, EF absence had a positive effect of nodulation while the effect of EB presence was almost negligible. Generally, many studies have clearly demonstrated that when legume hosts both rhizobia and AMF, plant growth, yield, and nitrogen nutrition generally are much greater than for plants inoculated either with rhizobia or AMF alone (Antunes and Goss 2005; de Varennes and Goss 2007; Meghvansi et al. 2008; Kaschuk et al. 2009). In fact, it has been suggested that mycorrhizae are a necessary precondition for

effective nodulation of many legumes (Crush, 1974). However, the higher carbon costs to plants of maintaining both fungal and bacterial symbionts may result in indirect antagonistic interactions between the two symbionts (Bethlenfalvay et al. 1985). This competition over plant carbon could explain the minimal effect on nodulation when all three microbes co-occurred e.g. the AM fungus, rhizobacteria and endophytes. However, a positive effect was reported when two out of three microbes remained especially in the absence of EF, perhaps because endophytic fungi have been reported to exhibit an extra antagonistic effect on AMF besides the antagonistic effect caused by carbon limitation. In this context, further study should focus on the role of nutrient fertility and endophyte role in plant soil feedback processes in the presence of AMF.

Our high-throughput sequencing identified a high frequency of *Ascomycota* phylum inside *T. repens* seeds, especially *Stemphylium* (28.4%), *Alternaria* (14.7%), *Epicoccum* (14.7%), and *Cladosporium* (9.8%). In fact, all of these abundant genera are considered as plant pathogens because they cause several diseases of crops depending on the species and the host plant specificity (Medina et al. 2019; Wolters et al. 2018; Chen et al. 2020; Griffiths et al. 2018). Yet, one of these common pathogens identified inside our seeds is *Epicoccum*, which is known as a genotypically and phenotypically highly variable species (Arenal et al. 2002). Moreover, several studies have demonstrated the antagonistic effect of members of *Epicoccum* against plant pathogenic agents for different crops (Larena et al. 2005; Koutb and Ali, 2010; Favaro et al. 2012; Kosawang et al. 2018) due to the high release of secondary metabolites, some of which have antifungal and antibacterial activities (Brown et al. 1987; Dzoyem et al. 2017). In fact, these results suggest a potential explanation for the increase of negative plant soil feedback by soil conditioning with fungal seed endophytes. This could be due to the accumulation of soil-borne pathogens, some of which were identified in the fungal endophytic microbiota of seeds. It is possible that such pathogens would move from the seeds into the soil during the conditioning phase and, thereafter, exhibited their negative effect on the response phase. These results align well with several previous studies suggesting that plant-soil negative feedback is due to the accumulation of pathogens in the soil (Cesarano et al. 2017). Pankhurst et al. (2005) suggested that the poor growth and yield decline of sugarcane (*Saccharum spp*.) grown in continuous monoculture is due to the presence of deleterious soil organisms. When the soil was treated, mainly by fungicides, the growth and yield of sugarcane increased in comparison with that in the untreated soil. However, for a better understanding of the role of soil biota in negative-plant soil feedback the evaluation of composition and changes in the entire soil and rhizosphere microbial community is a necessary step. In our study, the

application of an AM fungus along with the fungal endophytes during the conditioning phase did not help in shifting the plant-soil feedback. Similarly, Du Toit et al. (2018) reported no effect of *G. intraradices* as an AM fungus inoculant applied in furrows at seeding on pink rot in onions. Moreover, no effect of two commercially available AMF inoculants on the severity of *Stemphylium* leaf blight of two onion cultivars on mineral soil was found during the investigations of Ilyas, (2019). On the other hand, regarding bacterial endophytes, in our experiments high-throughput sequencing identified a high relative abundance of *Proteobacteria* and in particular, members of the *Enterobacteriaceae* family (>35%). Previous identifications have reported that most *Enterobacteriaceae* family members belong to plant growth-promoting rhizobacteria (Sivasakthi et al. 2013; Khalifa et al. 2016; Melo et al. 2016; Barrao et al. 2017). Most combined applications of plant growth-promoting rhizobacteria and AMF are used to increase the yields of crops (Mäder et al. 2011), fruit quality (Ordookhani et al. 2010) to improve the nutrient use efficiency of fertilizers, and to allow reduced application of chemical fertilizers (Adesemoye et al. 2009). However, and as stated previously, the higher C costs to plants of maintaining both fungal and bacterial symbionts may result in indirect antagonistic interactions between the two symbionts (Bethlenfalvay et al. 1985), which could be the explanation for the generated negative feedback when these bacteria coexisted with AMF during the response phase.

3.6 Conclusion

Our study demonstrated that the seed microbiome plays a role in determining the direction of plant-soil feedback. While seed fungal endophytes generated a strong conspecific negative feedback, seed bacterial endophytes proved to shift feedback from negative to positive or vice versa. The simultaneous occurrence of both types of seed endophytes with the AM fungus generated negative plant soil feedback. To our knowledge, this is the first time that the role of seed bacterial endophytes in conspecific plant-soil feedback was investigated, and it demonstrated that the AM fungus completely reversed the direction of the feedback effect on bacterial endophyte-associated plants. Furthermore, high-throughput sequencing illustrated that the negative plant-soil feedback generated by seed fungal endophytes may be due to the presence of abundant pathogens in the seeds, which would likely have emerged into the soil and escaped the AM fungus control. However, the positive feedback created by seed bacterial endophytes in the absence of the AM fungus likely was due to abundance of plant growth-promoting rhizobacteria among the seed endophytes. Their negative interaction with the AM

fungus may have been caused by their competition with seed fungal endophytes and the AM fungus for the limited organic carbon offered by the host plant. Subsequent studies are needed to understand fully the mechanisms of interaction between both types of seed endophytes, as well as their interactions with AMF in the control of conspecific plant-soil feedback processes.

4 Chapter 4: Specific microbiome signatures depend mainly on the chemical properties of shrub's litter within a Mediterranean shrubland

4.1 Abstract

Shrub encroachment (SE) is a phenomenon in which grasses and herbaceous vegetation are replaced by woody shrubs. Many previous studies have highlighted the effects of SE on soil respiration rates and nutrient storage, but little is known about impacts on soil microbiota. While previous work considered shrubs to be non-species specific or as a single intervening species, we selected an *Ampelodemsos mauritanicus* grassland and six coexisting shrubs (i.e. *Pistacia lentiscus* L., *Juniperus phoenicea* L., *Myrtus communis* L., *Rosmarinus officinalis* L., *Olea europaea* L., and *Euphorbia dendroides* L.) to investigate the effects of their encroachment on soil microbiota. We used high-throughput sequencing, coupled with soil chemical analyses and litter using ^{13}C CPMAS NMR spectroscopy. Results showed a strong influence of shrub species on bacterial and fungal community diversity, species richness and overall community composition in the soil. Litter chemistry was dominated by O-alkyl-C, with the highest content in *Ampelodesmos* and *E. dendroides*, but richer of aromatic C in *P. lentiscus* and *R. officinalis*. Bacterial diversity was highest under *J. phoenicea* and *E. dendroides*, while lowest under *R. officinalis* and grassland. Conversely, fungal diversity was highest under *O. europaea* and *E. dendroides*, while lowest under *M. communis* and grassland. Moreover, soil C and N contents were highest under *O. europaea*, *P. lentiscus* and *M. communis* compared to the other shrub species. In addition, grassland and *R. officinalis* had the highest Fe content. Structural equation model (SEM) analysis ascertained that the shifts of bacterial and fungal community composition and diversity were closely related with the changes of litter and soil chemical properties. Our results suggest that the individual effect of each shrub on the grassland matrix depends mainly on the chemical properties of the shrub litter, which alters the chemical profile of the soil and, in cascade, shapes the associated microbiota.

Keywords: shrub encroachment, grassland, Mediterranean, microbial community, litter chemistry; ^{13}C CPMAS NMR spectroscopy.

4.2 Introduction

Grassland ecosystems occupy approximately 41% of the Earth's land surface (Reynolds et al., 2007), and play an important role in global biogeochemical, hydrological, and energy cycles (Huang et al., 2018). However, in 10 - 20% of these ecosystems, shrub encroachment (SE) is occurring (Reynolds et al., 2007). SE is a phenomenon in which grasses and herbaceous vegetation are replaced by woody shrubs (Sankaran et al., 2004). SE is a form of land cover change that is widespread in arid and semi-arid grassland ecosystems (Eldridge et al., 2011). This phenomenon has been shown, thus, to alter the landscape, microclimate, and above- and below-ground biological processes (Dong et al., 2014). Several studies have suggested many possible causes for SE, including the increase in atmospheric CO_2 (Wigley et al., 2010), climate change (D'Odorico et al., 2010), nitrogen deposition (Kochy & Wilson, 2001), changes in fire regime (Van Auken, 2000), and overgrazing (Briggs et al., 2005).

The global encroachment at the expense of grasses is predicted to increase in the next years, as woody plants have multiplied, in many parts of the world, over the past 100 years (Kulmatiski & Beard, 2013). Woody plant encroachment is often a conservation concern (Van Auken, 2000) because it alters functions and processes of grassland ecosystems such as total primary productivity, decomposition rates, nutrient availability, and soil carbon dynamics (Eldridge et al. 2011). The resulting resource distribution, also called “islands of fertility” (Schlesinger et al., 1990), could favour the growth of other woody species because of the shrub nurse effect (Callaway, 2007), thus creating a positive feedback that could lead to an irreversible woody encroachment process (Du et al., 2016). The formation of fertility islands under woody plant canopies involves several ecological processes. First, the extensive root system of woody plants is able to extract nutrients from the depth of the subsoil and the interspaces that are deposited under the canopy through litterfall (Gherardi et al., 2013). The formation of island of fertility is especially evident under nitrogen-fixing woody species that accumulate large amount of nitrogen (N) and phosphorus (P) in soil through litterfall (Facelli & Brock, 2000). The formation of island of fertility is dependent on litterfall amount, litter chemical traits, litter decay rate and associated accumulation of soil organic carbon. For example, Stinca et al., (2015) reported that colonisation of the nitrogen-fixing *Genista aetnensis* over bare volcanic soil resulted in a sharp increase in organic carbon (OC), N and P stocks in the topsoil in few decades. On the other hand, invasion of *Juniperus virginiana* into lower-lying grasslands in the western USA has been shown to alter the amount and distribution of C and N stocks in soil and plants (McKinley & Blair, 2008). As a result, the soil under the

canopy of woody plants becomes the preferred site for plants, animals and microorganisms, whose metabolism increases soil OC and N and further enriches soils in the understory (Dean et al., 1999). In addition, shrub canopy reduces wind speed, allowing atmospheric dust, wind- and water-transported nutrients, detritus, and seeds being accumulated beneath woody canopies (Eldridge et al., 2011). As a result, in further fertile island effect enhancement (Reynolds et al., 1999).

Understanding the consequences of SE is important. However, most studies on the effects of SE have focused mainly on vegetation (Hu et al., 2015), soil chemical cycling (Eldridge et al., 2015), and microbial biomass and enzymatic activities (Eldridge et al., 2015). Instead, little attention has been paid to the effects on soil microbiota composition and diversity (Yannarell et al., 2014). Plants are known to influence soil microbial communities through both the quantity and quality of their aboveground litter and through root exudates released into the soil that feed heterotrophic soil microorganisms, leading to overall shifts in the community composition of soil (Wallenstein et al., 2007). In this context, SE has been shown to significantly increase bacterial biomass (Yannarell et al., 2014) and alter fungal community composition (Bragazza et al., 2015). Therefore, dynamic and complex feedback mechanisms exist between aboveground vegetation and the belowground microbial community (Xiang et al., 2014). Moreover, microbial species are actors in the plant-soil feedback that can alter the outcome of plant competition and drive the process of plant community succession (Idbella et al., 2021). For this reason, SE may have lasting consequences for grassland ecosystem restoration and management, as shifts in microbiota composition may facilitate long-term succession from grassland to shrub/forest ecosystems (Yannarell et al., 2014).

As for the study of the influence of SE on the composition of the soil microbial community, very little evidence was found in the literature, since they all consider shrubs as non-species specific or as a single encroached plant type. For example, Xiang et al., (2019) showed that the encroachment of *Caragana microphylla* into a grassland dominated by *Cleistogenes songorica* induced significant changes in soil bacterial community composition. Similarly, Ding et al., (2020) showed that the encroachment by *Vaccinium fragile* into a grassland dominated by *Eulalia pallens* significantly restructured the diversity and composition of soil bacterial and fungal communities. On the other hand, Yannarell et al., (2014) showed that the encroachment by four different species on the grassland Remnant Hill Prairies significantly altered both bacterial and fungal communities without selecting species-specific signatures under the canopy of each woody plant. However, most of the previous studies compared only a single woody species with the surrounding open vegetation. Here, our study

aimed to investigate the influence of six coexisting Mediterranean shrubs (i.e. *Pistacia lentiscus* L., *Juniperus phoenicea* L., *Myrtus communis* L., *Rosmarinus officinalis* L., *Olea europaea* L., and *Euphorbia dendroides* L.), over *Ampelodemsos mauritanicus* L. grassland, on the soil microbiota. Specifically, we combined soil and litter chemistry analyses with next-generation sequencing techniques to determine how the soil microbiota is shaped by the canopy of Mediterranean shrubs. Our hypothesis states that litter chemistry is different between shrubs, resulting in a specific microbial fingerprint. This specific effect is thought to be derived from the specific chemical characteristics of the litter of the shrubs as it falls and decomposes (De Marco et al., 2011), in addition to the plants' root exudates (Shi et al. 2011), leading to different changes in soil chemistry and also microbial composition. The aim of this study was therefore to provide basic information and novel insights into the environmental selection of soil microbial communities by each of the most abundant species-specific shrubs in a Mediterranean SE ecosystem. Specific aims were:

i. to assess the "island of fertility" effect under the canopy of different Mediterranean shrubs;

ii. to describe the bacterial and fungal microbiota associated with the different shrub species;

iii. to explore the link between soil chemistry and bacterial and fungal microbiota.

4.3 Material and Methods

4.3.1 Study site description

The study was conducted in Cape Palinuro shrubland site (40°01'35 "N 15°16'30 "E), located in southwestern Italy, about 40 miles southwest of the city of Salerno (Fig. 1). This area is located in a Mediterranean climate characterised by mild winter and hot and dry summer. Vegetation is adapted to dry summers and it is fragrant and oily, making it susceptible to fire. The elevation of the study area is 185 m a.s.l., and the average annual temperature is 16.7°C. In winter, temperatures average is 13.3°C during the day and drop to 7.9°C overnight; in spring, temperatures reach 17.6°C, mostly in the afternoon, while during the night-time it drops to 11.2°C; in summer, average maximum temperature is 27.1°C and average minimum is 19.7°C. The average annual precipitation is 789.8 mm. The site is characterized with limestone rocks overlying clay soils with abundant rock outcrops.

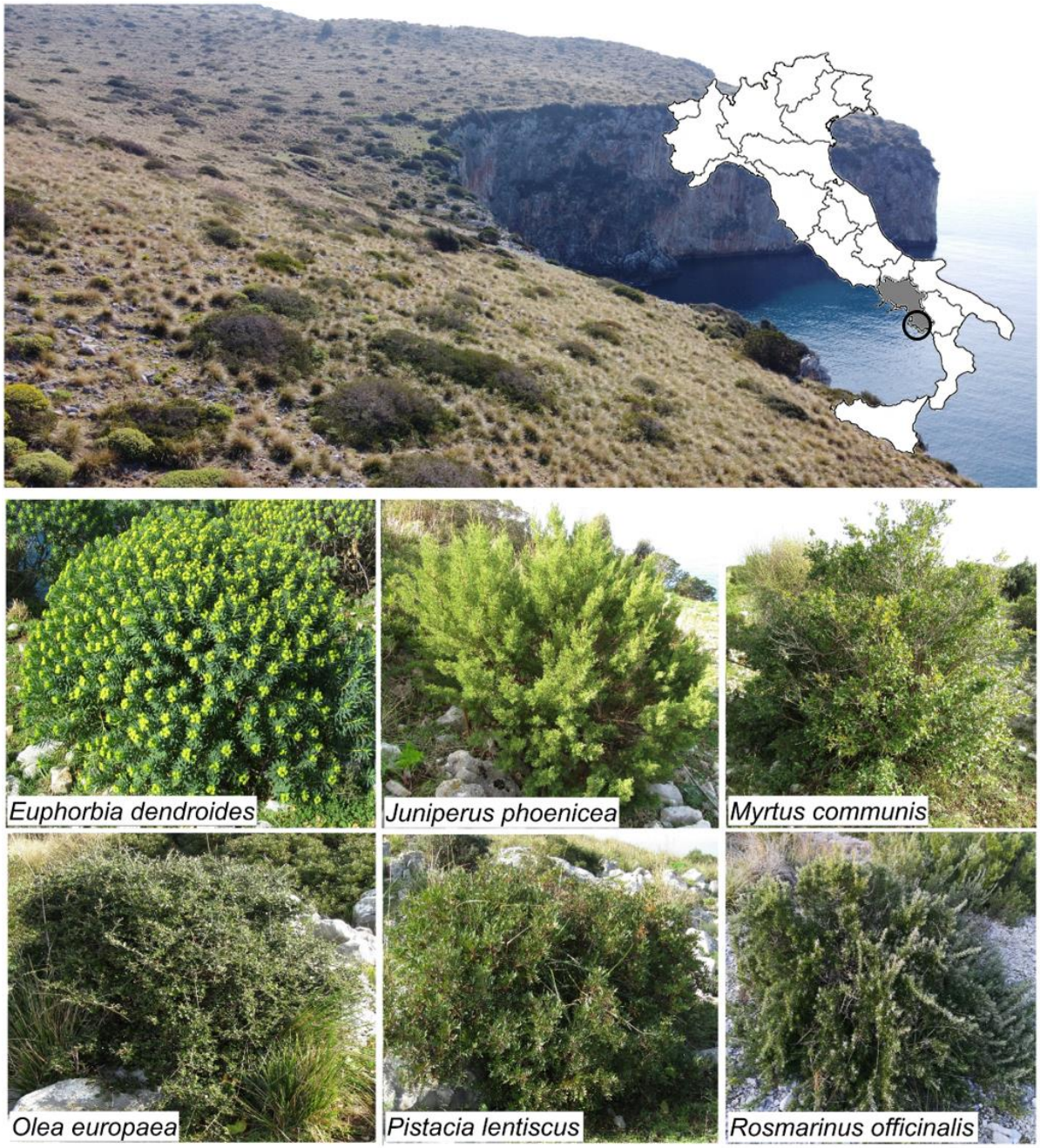

Fig 1. Sampling site of Capo Palinuro located in Southern Italy in Campania region (black circle on the map) on the grassland matrix, at the top of the figure, dominated by *Ampelodesmos mauritanicus* in which the studied shrubs have encroached; followed by the six encroached shrubs studied at the shrubland.

4.3.2 Soil sampling

Within the study area, six shrub species were selected for soil sampling, including five evergreens: *Pistacia lentiscus* L., *Juniperus phoenicea* L., *Myrtus communis* L., *Rosmarinus officinalis* L. and *Olea europaea* L., and one deciduous: *Euphorbia dendroides* L. (Fig. 1). Soil

samples were also collected in the grassland soil dominated by *Ampelodesmos mauritanicus* L., a perennial, fire-prone tall grass that dominates in the matrix between shrubs (Incerti et al., 2013). In April 2019, for each shrub species, three individual shrubs were randomly selected. Under each individual, four soil samples were collected from different positions located between the shrub trunk and the periphery of the canopy and pooled. Overall, 21 samples were collected: 7 plant types (6 samples for 6 shrub species + one sample for the grassland) with 3 replicates for each plant type. Shrub's size, based on the crown diameter, was 135 ± 22 cm for *E. dendroides*, 163 ± 74 cm for *O. europaea*, 173 ± 59 cm for *P. lentiscus*, 126 ± 9 cm for *J. phoenicea*, 164 ± 8 cm for *R. officinalis*, and 117 ± 11 cm for *M. communis*. Soil samples were collected by a 5 cm diameter soil corer, at a depth of 10 cm after removal of aboveground litter. Subsequently, soil was pooled and sieved (2 mm mesh) on site resulting in a single composite sample for each shrub replicate. The samples were stored in sterile plastic bags and labelled. Before sampling operation, the soil corer was thoroughly cleaned and sterilised to avoid between samples contamination. After collection, samples were then divided into two fractions: one fraction was kept at 4°C to investigate soil chemical properties; the other fraction was stored at −20°C and used for molecular analysis.

4.3.3 Litter collection and chemical analysis

Under each shrub species, leaf litter was collected with net traps during the period of maximum leaf fall from three randomly selected individuals of each shrub species. Litter was collected from the same individuals under which the soil was collected. Freshly collected litters were dried in a ventilated chamber at 30°C until they reached a constant weight and then stored at room temperature. Litters were then characterized for total C and N content by flash combustion of microsamples (5 mg of litter) using a CN soil elemental analyser (Flash EA2000 Thermo). Proximate cellulose and lignin content were quantified as acid-hydrolysable fraction and acid-unhydrolyzable materials, respectively (Gessner, 2005). In addition, leaf materials were characterized by ^{13}C cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) (Kögel-Knabner, 2002) obtained in the solid-state and under the same conditions, which allowed a comparative analysis of the resulting spectra. The spectrometer used was a Bruker AV -300 equipped with a 4 mm wide-bore MAS probe (Bonanomi et al., 2013). The spectral ranges and corresponding C types were identified as described by Bonanomi et al., (2013): 0-45 ppm = alkyl-C (characteristic of lipid waxes, cutins and microbial products); 46-60 ppm = methoxyl- and N-alkyl-C (characteristic of amino acids and lignin components); 61-90 ppm = O-alkyl-C (characteristic of carbohydrates and polysaccharides);

91-110 ppm = di-O-alkyl-C (anomeric C1 of celluloses, tannin and lignin components); 111-140 ppm = H- and C-substituted aromatic C (mainly associated with polyphenols, lignin and tannin components); 141-160 ppm O-substituted aromatic C (phenolic and O-aryl-C, characteristic of phenols, lignin and tannin components); 161-190 ppm carboxyl-C (characteristic of organic acids, amides, esters).

4.3.4 Soil chemical analyses

Soil samples were dried in a ventilated chamber at room temperature until a constant weight was reached. The soil was analyzed for 16 parameters i.e., total organic carbon (OC), pH, total nitrogen, and macro- and micronutrients important for plant growth. Specifically, the following parameters were measured: soil electrical conductivity (EC) and pH, were determined in 1:5 and 1:2.5 soil-water suspensions, using a conductivity meter and a pH meter, respectively (Czekała et al., 2016). Total nitrogen was determined by the Kjeldhal method (Czekała et al., 2016), while phosphorus was assessed by the molybdovanadate phosphate method (AOAC, 1990). Water content and organic matter content were determined by weight loss at 105°C for 24 h and 550°C for 8 h, respectively (Silva et al., 2014). Potassium, magnesium, iron, manganese, calcium, sodium, copper and zinc were determined by flame atomic absorption spectroscopy (Peters et al., 2003). Total limestone is determined by the weight method against a strong acid, the attack of the limestone leads to a gas release of CO_2, the volume of which is measured (LANO: NF ISO 10693). Finally, the chloride content (Cl) in the soil was determined by the volumetric method described by Meldrum and Forbes (1928).

4.3.5 Soil DNA extraction and amplification

The microbiome of homogenized soil samples under each shrub species was analyzed by Illumina high-throughput sequencing. The DNeasy PowerSoil kit (Qiagen) was used to extract the microbial DNA from 2 g of each homogenized soil. Bacterial and fungal diversity were assessed by high-throughput sequencing of the amplified V3-V4 regions of the 16S rRNA gene (~460 bp) and ITS1-2 (~300 bp). PCR was carried out with primers S-D-Bact-0341-b- S-17/S-D-Bact0785-a-A-21 (Berni Canani et al., 2017) and BITS1fw/ B58S3- ITS2rev (Bokulich & Mills, 2013) using conditions reported in the original studies. For bacterial primers S-D-Bact-0341-b-S-17 (5’ -CCTACG GGNGGCWGCAG-3’) and S-D-Bact-0785-a-A-21 (5’ -GAC TACHVGGGTATCTAATCC-3’), PCR conditions were: 25 cycles of 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 72°C for 5 min and held at 4°C. For fungal primers

BITS1fw (5'-ACCTGCGGARGGATCA-3') and B58S3-ITS2rev (5'-GAGATCCRTTGYTRAAAGTT-3') PCR conditions were: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension of 72°C for 5 min. PCR products were purified with the Agencourt AMPure beads (Beckman Coulter, Milan, IT) and quantified using an AF2200 Plate Reader (Eppendorf, Milan, IT). Equimolar pools were obtained and sequencing was carried out on an Illumina MiSeq platform, yielding to 2× 250 bp, paired-end reads.

4.3.6 Sequence data analysis

Demultiplexed fastq files were processed using the DADA2 package (version 1.16.0 pipeline) (Callahan et al., 2016) in R software (4.0.4) (Team, 2016). DADA2 provides better taxonomic resolution than other methods because it retains unique sequences and calculates sequencing error rates rather than clustering to 97% similarity (Hugerth & Andersson, 2017). The resulting taxonomic units are referred to as amplicon sequence variants (ASVs) rather than operational taxonomic units (OTUs). For bacterial sequences, forward and reverse reads were trimmed to 250 bp, primer sequences were removed from all reads, and filter parameters used were the following: maxN = 0, maxEE for both reads = 2, truncQ = 2 (MaxEE corresponds to the maximum expected errors, TruncQ represents the parameter that truncates reads on the first occurrence of a quality score less than or equal to two, and MaxN is the maximum number of 'N' bases accepted). Error rates were estimated by learnErrors using nearly 4 million reads. Sequences were dereplicated using derepFastq with default parameters and exact sequence variants were resolved using the dada algorithm. The RemoveBimeraDenovo function was then used to remove chimeric sequences. For fungal sequences, the pipeline was pre-empted by a preliminary step of trimming adapter sequences and low-quality ends (<Q20) using Cutadapt software (Martin, 2011). For both the bacterial and fungal datasets, reads with more than three errors in the forward reads and five errors in the reverse reads were removed. Taxonomy was then assigned using assignTaxonomy based on the SILVA (v132) and UNITE (v7) databases for bacterial and fungal communities, respectively (Quast et al., 2013; Nilsson et al., 2019). *Chloroplast* and *Streptophyta* contaminants and singleton ASVs were removed, and relative abundances of the other taxa were recalculated.

4.3.7 Statistical analysis and data visualization

Plotting was performed using PRIMER 7 software (Primer-E Ltd, Plymouth; UK). Alpha diversity metrics, i.e. Margalef species richness index and Shannon index, were calculated.

Heatmaps were created to assess variation in community composition at lowest taxonomic levels. Heatplots were used for clustering variables according to the results of an index of association similarity and for clustering samples according to Bray-Curtis dissimilarity. The 50 most abundant taxa in the fungal and bacterial communities are shown in the heatplots. A resemblance matrix calculated based on Bray-Curtis dissimilarity was used to perform non-metric multidimensional scaling (nMDS) to assess variation in species composition under the different investigated species for the bacterial and fungal communities. The significance of changes in composition between the two microbial communities was tested by PERMANOVA (999 permutations), using the shrub species as fixed factor. The significance of variation in the alpha diversity metrics of the two microbial communities was assessed along with the soil and litter chemical characteristics using the ANOVA test, and the means were pairwise separated using the *post-hoc* Tukey test for more details about the significance level between samples. The level of significant differences was assessed at $P< 0.05$. All statistical analyses were performed using STATISTICA 13.3 software.

Furthermore, we analysed functional group variation for the fungal community, identifying putative fungal functional groups as well as their trophic modes using FUNGuild (Nguyen et al., 2016). The core microbiome was identified by constructing Venn diagrams for 7 sets (i.e., 6 shrub species + the grass) for bacterial and fungal communities using R software and the VennDiagram package (Chen & Boutros, 2011). Structural equation model (SEM) was constructed to investigate the direct and indirect effects of litter and soil chemical properties on microbial diversity and composition. Diversity was characterized by the Shannon index and composition by the first axis of the PCoA using the Bray-Curtis dissimilarity matrix. Based on a priori and theoretical knowledge, we assumed a conceptual model that differences in litter chemistry affect soil chemical parameters, which affects in cascade the microbial diversity and composition. Maximum likelihood estimation method was used to compare the SEM with observations. Model adequacy was determined by chi-square tests, goodness-of-fit index (GFI), Akaike Information Criteria (AIC) and root square mean errors of approximation (RMSEA). Adequate model fits were indicated by a non-significant chi-square test ($P > 0.05$), high GFI, low AIC, and low RMSEA (Grace, 2006). SEM analysis was performed using AMOS 26.0 (Amos Development Corporation, Meadville, PA, USA).

4.4 Results

4.4.1 Litter and soil chemistry

Leaf litter chemistry varied largely among shrub species and the grass *A. mauritanicus* (Table 1). *A. mauritanicus* had the highest cellulose content and C/N and lignin/N ratios, while it had the lowest N content and relatively low lignin content. On the other hand, the highest lignin content was found in *R. officinalis* litter, which also had high N content and the lowest cellulose content. *P. lentiscus* had relatively high contents of all proximate parameters except N content, which was relatively low. *J. phoenicea* had very low N content, and thus the highest C/N and lignin/N ratios. *E. dendroides* leaf litter, on the other hand, had the highest N content and the lowest C/N and lignin/N ratios. Finally, *M. communis* leaf litter recorded the lowest lignin content among all other shrub species and thus a very low lignin/N ratio.

The ^{13}C-CPMAS NMR data showed that *E. dendroides* leaf litter had the highest content of carboxylic C, followed by *R. officinalis* and *M. communis*; while the others, including *A. mauritanicus*, had significantly low values. The O-substituted aromatic C fraction was highest in *P. lentiscus* and lowest in *A. mauritanicus* and *E. dendroides*. Moreover, *P. lentiscus* and *R. officinalis* showed the highest H-C substituted aromatic C content while the lowest was found in *E. dendroides*. However, *A. mauritanicus* and *R. officinalis* had the highest di-O-alkyl C and O-alkyl C fractions, while the lowest methoxyl C and alkyl C fractions were recorded for *A. mauritanicus* alone. In addition, *E. dendroides* and *O. europaea* had the highest methoxyl C fraction, and *J. phoenicea* was the one that contained the highest alkyl C fraction content instead.

Soil chemical parameters showed significant variation among shrub species (Table 2). Clustering of the shrubs, by D1 Euclidean distance, based on chemical parameters resulted in five clusters for the six studied shrubs and the grass, *A. mauritanicus*, separating *E. dendroides*, *M. communis*, *J. phoenicea*, Grassland- *R. officinalis* and *P. lentiscus* - *O. europaea* (Fig. S1). In details, *E. dendroides* and *M. communis* showed the highest soil pH compared to grassland and other shrubs. In addition, *E. dendroides* soil had the highest total limestone by a maximum of 4.11% and soil potassium by a maximum of 1.27 g/kg. Electrical conductivity, chlorides and sodium were higher under *O. europaea* and *M. communis*, while significantly lower values were measured under the rest of the shrubs, with an exception for sodium under *J. phoenicea*. The soil OC and total N contents were higher under *M. communis*, *P. lentiscus* and *O. europaea* than under the rest of the shrubs. In addition, the highest P and Mg contents were found in the soils under *E. dendroides* and *M. communis* species. The distribution of Ca, Cu, Zn and Mn was different among the shrub species. Fe content was found to be significantly higher only in

the grassland and under *R. officinalis* compared to other shrubs, while the lowest value was found under *E. dendroides*.

Table 1. Chemical traits of leaf litter of different plant species. Values are mean of three replicates. Different letters within each column indicate significant difference (Duncan test, $p<0.05$).

		dendroides	phoenicea	communis	europaea	lentiscus	officinalis	mauritanicus
Elemental and proximate parameters	Cellulose (%)	14.2b	13.0c	15.8bc	10.6d	17.9b	8.6d	27.3a
	Lignin (%)	13.2d	23.8b	10.6e	16.3c	23.9b	41.4a	18.1c
	N (%)	2.1a	0.7d	1.2c	1.7b	1.1c	1.9a	0.5d
	C / N	20.5d	69.3a	41.1b	31.2c	45.4b	22.8d	77.6a
	Lignin / N	6.3d	33.0a	8.7d	16.3c	21.7b	21.6b	34.5a
^{13}C-CPMAS NMR-derived parameters	Carboxylic C	9.8a	4.4c	6.2b	5.6c	5.3c	6.9b	4.8c
	O-aroma C	4.4d	4.8cd	5.7c	3.6e	9.3a	7.4b	4.0d
	di-O-alkyl C	10.8bc	9.7cd	11.4b	8.9d	12.6b	11.1a	14.9a
	O-alkyl C	44.8b	38.4c	42.4b	39.1c	34.7c	37.5c	55.7a
	Methoxyl C	8.1a	6.2b	6.2b	8.3a	4.5c	5.6bc	4.6c
	Alkyl C	17.4d	27.5a	19.2c	24.9b	21.5bc	19.9c	6.7e

4.4.2 Microbial diversity

Significant variation in Shannon diversity and species richness was found for bacterial and fungal diversity. Bacterial species richness was significantly higher in the soil under *E. dendroides* compared to the soil under *R. officinalis*, while slight, although not significant, variations were observed for the other shrub species (Fig. 2a). The Shannon index of bacteria was significantly higher in the soil under *J. phoenicea* and *E. dendroides* compared to *R. officinalis* and the grassland. On the other hand, no significant variation was found in fungal species richness, while the *O. europaea* showed the highest Shannon diversity index of the soil fungi, which showed a statistical significant difference compared to *M. communis* and grassland soils (Fig. 2b).

Table 2. Chemical analysis of the soils collected under the canopy of plant species in the studied shrubland as well as the grassland. Different letters within each parameter indicate significant differences (Duncan test, $p < 0.05$).

Parameters	Grassland	phoenicea	officinalis	lentiscus	europaea	communis	dendroides
pH	5.62c	6.00c	5.82c	6.32b	6.34b	6.74a	6.93a
Water content (%)	5.82c	11.19b	6.31c	9.13b	9.42b	10.46b	16.99a
Total limestone (%)	0.87b	1.75b	1.39b	0.82b	0.67b	1.11b	4.11a
Electrical conductivity (mS/cm)	0.31b	0.26b	0.21b	0.34b	0.42a	0.47a	0.31b
Chlorides Cl (g/Kg)	0.21a	0.15bc	0.13bc	0.18b	0.278a	0.30a	0.087c
Sodium Na_2O (g/Kg)	0.25b	0.40a	0.19c	0.31b	0.43a	0.43a	0.26b
Organic Carbon (%)	12.48b	11.90b	11.53b	16.6a	16.27a	16.17a	9.80b
Total Nitrogen (%)	0.50b	0.47b	0.42b	0.77a	0.85a	0.99a	0.70a
P (mg/Kg)	20.47b	21.73b	12.55c	20.6b	20.88b	35.20a	39.89a
K (g/kg)	0.35c	0.65b	0.44bc	0.51b	0.53b	0.55b	1.27a
Mg (g/Kg)	0.57b	0.91a	0.46b	0.89ab	0.97a	1.20a	1.32a
Ca (g/Kg)	3.76b	3.91b	2.63b	9.01a	8.65a	10.92a	8.40a
Cu (mg/Kg)	1.37b	1.62b	1.43b	2.04a	2.52a	1.87a	2.05a
Zn (mg/Kg)	8.08c	3.96d	5.53d	17.95b	18.68ab	22.59a	4.69d
Mn (mg/Kg)	26.66b	30.84ab	25.48b	43.98a	39.82a	27.77b	13.29c
Fe (mg/Kg)	56.78a	29.80b	44.57a	23.25b	30.00b	23.23b	5.21c

4.4.3 Bacterial community composition

At the phylum level, considerable significant variation was found among shrubs and the grassland soils in the bacterial community (Fig. 3a, Fig. S2, Table S1, S2). The soil under all shrubs harboured mainly *Actinobacteria*, ranging from 30.1% in *R. officinalis* to 40.3% in *O. europaea*, except for *P. lentiscus*, which harboured equally *Actinobacteria* and *Proteobacteria*, with a proportion of 25.4% each. On the other hand, the lowest abundance of *Proteobacteria* was found in the grassland soil at 15.7% compared to 20.9% – 25.4% among the other soils. *Acidobacteria*, on the other hand, was most abundant in the grassland soil at 18.5% followed by *P. lentiscus* at 15.8% and less than 10% among the other soils. In addition, the highest levels of *Verrucomicorbia* were found in *M. communis* soil (13.2%), followed by *P. lentiscus* (8.7%) and the remaining soils with less than 5%. On the contrary, *Planctomycetes* was higher in *R. officinalis* soil (20.2%) compared to the other soils (< 13%).

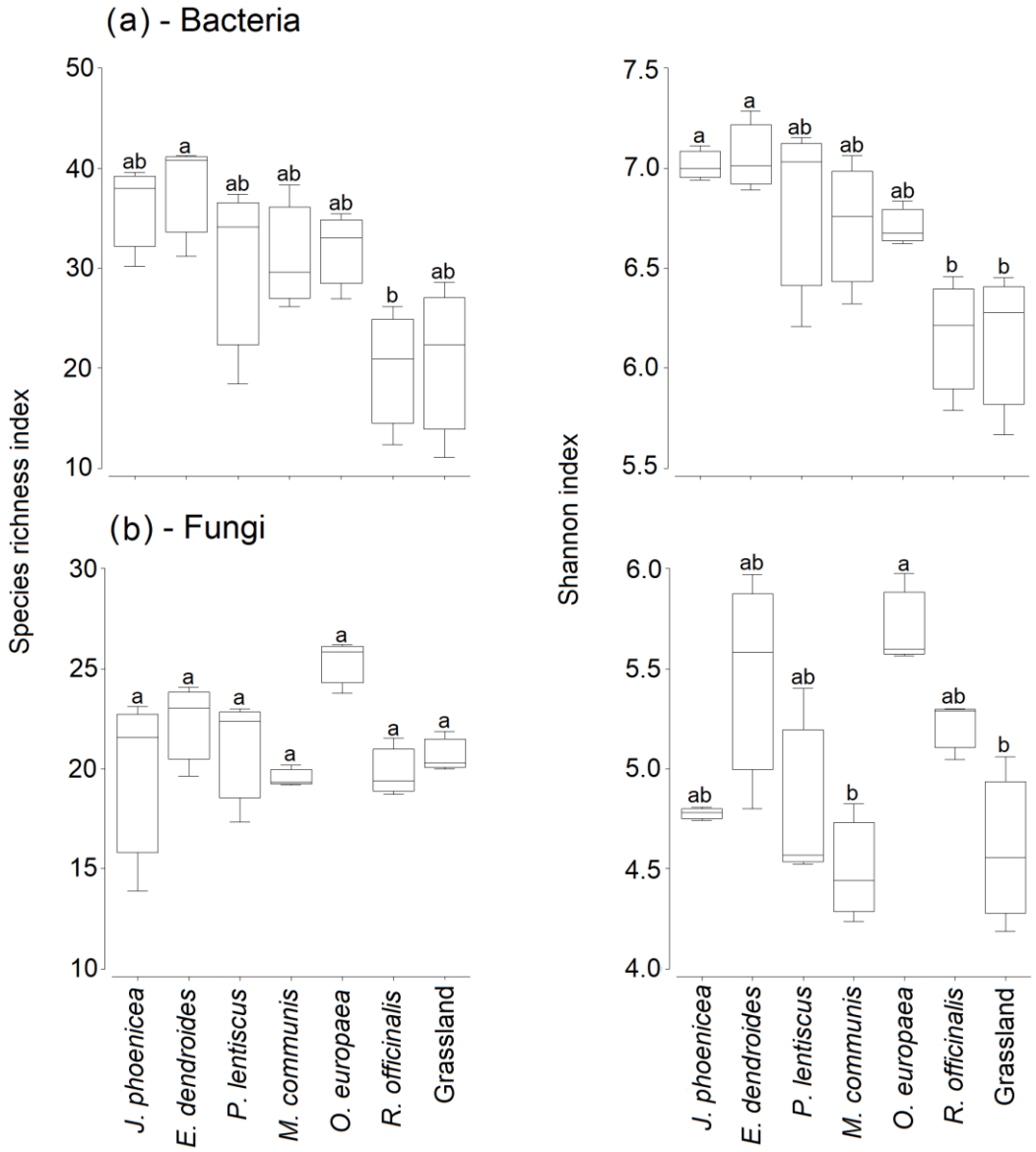

Fig 2. Box plots showing the variation in the Shannon diversity and species richness indices for bacterial (A) and fungal (B) communities under each plant species across the shrubland of Capo Palinuro. Different letters indicate significant ($p < 0.05$) differences in the indices under different shrub species. The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median, whiskers above and below the boxplot indicate inter-quartile range.

The Venn diagram (Fig. 4a) confirmed that the soil under *E. dendroides*, *J. phoenicea*, and *P. lentiscus* are the ones that include the highest number of unique ASVs, where they attained 47, 34, and 31 ASVs, respectively. While the lowest number of unique ASVs was found in the soil under *R. officinalis* and *O. europaea*. However, 219 ASVs were determined as the core bacterial microbiota of the studied microbiomes.

At the lowest taxonomic level (Fig. 5), the clustering of the shrub species, based on the 50 most frequent ASVs, is highly distinct using the Bray-Curtis similarity index across different shrubs and the grassland soils (p = 0.001; Table S2), where it revealed three main different clusters for the six shrubs and the grassland. A first group (cluster A) includes *P. lentiscus* soil characterized by a high abundance of *Acidobacteria* subgroup_6. The second group (cluster B) includes *M. communis*, *J. phoenicea*, *O. europaea*, *E. dendroides* and grassland. The main driving ASVs of cluster B were *Rubrobacter*, which was abundant in grassland and *O. europaea* soils, and *Candidatus Udaeobacter*, which was abundant only in *M. communis* soil, followed by grassland. Finally, a group (cluster C) that included *R. officinalis* and characterized by *Rubrobacter*, which was highly abundant, and *Isosphaeraceae*.

The nMDS analysis of the bacterial community in relation to the chemical parameters (Fig. S4a) showed a strong positive correlation between Fe and the ordination of the grassland samples. However, *R. officinalis* and *O. europaea* were positively correlated with Cu, Na, total N, Mg, and Cl and negatively correlated with OC, total limestone, P, Mn, Ca, and Zn; with an opposite pattern for the samples ordination of *P. lentiscus* and *M. communis*, which was positively correlated with OC, P, and total limestone and negatively correlated with total N, Na, and Mg. Finally, *J. phoenicea* and *E. dendroides* showed a high negative correlation with Fe, which was the main reason for their samples ordination.

4.4.4 Fungal community composition

The fungal community showed a clear difference among the shrub species and in comparison with the grassland (Fig. 3b, Fig. S3, Table S1, S2). Specifically, all the soils studied were dominated by the phylum *Ascomycota*, with abundance ranging from 60.5% in the grassland soil to 80.3% in *M. communis* and *R. officinalis*. However, the highest proportion of the phylum *Basidiomycota* was found in the grassland soil (25.1%), followed by *J. phoenicea*, *E. dendroides* and *M. communis* with ~ 16%, while in the other soils their abundance did not exceed 10%. On the other hand, the phylum *Chytridiomycota* was found with an abundance of 5.7% in the soil under *O. europaea*, and less than 2% in the rest of the soils. In addition, *P. lentiscus* showed exclusivity in harbouring the *Mortierellomycota* phylum (15.4%), followed by *E. dendroides* (10.9%), while it was almost absent under the *R. officinalis* soil. Finally, the phylum *Zoopagomycota* was present in only 2.4% of the *P. lentiscus* soil.

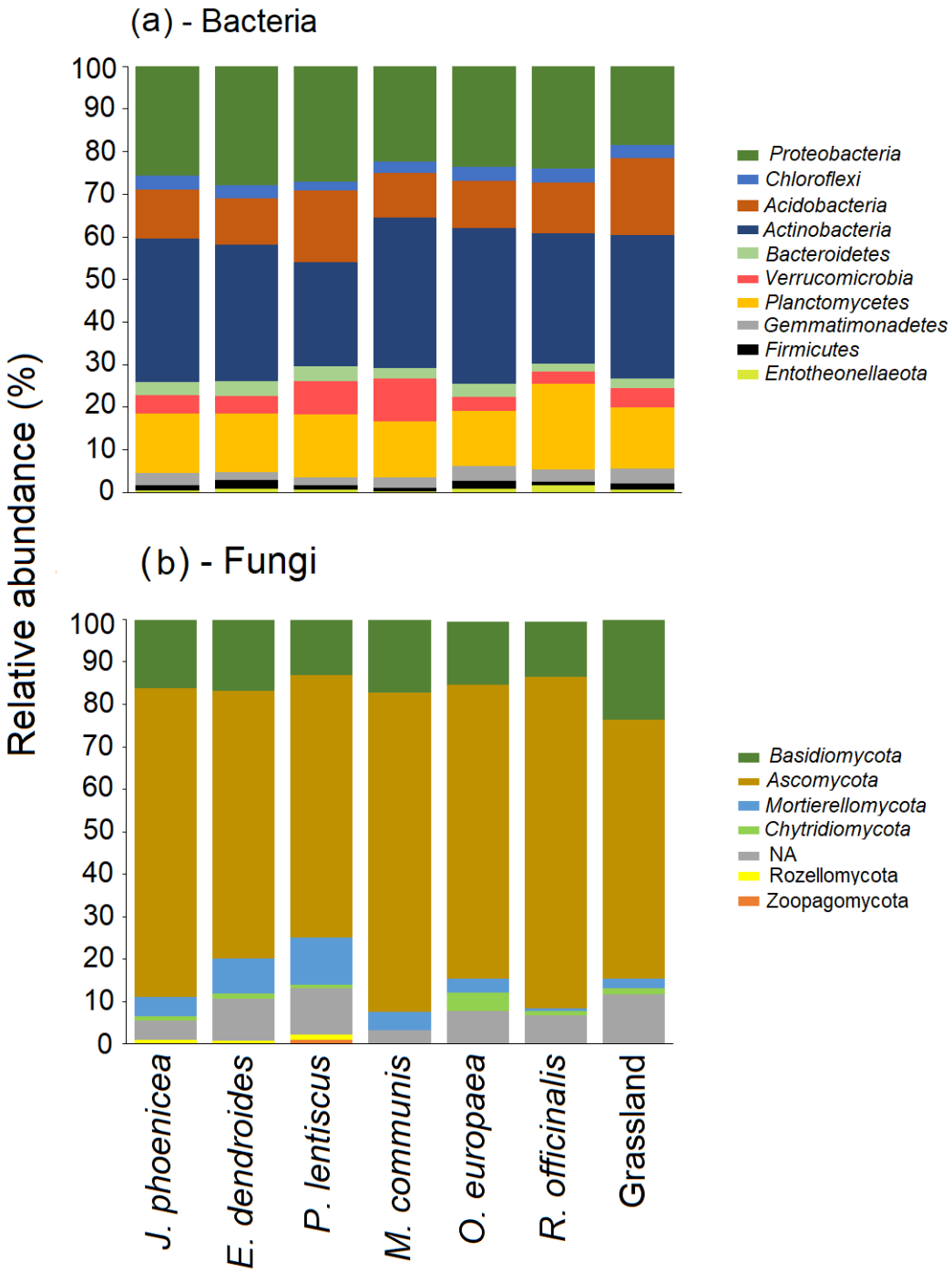

Fig 3. The relative abundance of various bacterial (A) and fungal (B) phyla in the soil of each plant across the shrubland and grassland.

The Venn diagram showed that all the soils have enclosed a high amount of exclusive fungal ASVs compared to the bacteria (Fig. 4b). The highest number of unique ASVs was found under the grassland (i.e., 46) and the lowest was found under *E. dendroides* (21) and *M. communis* (22). However, the core microbiota, responsible for the most frequent ASVs, was represented by 115 ASVs.

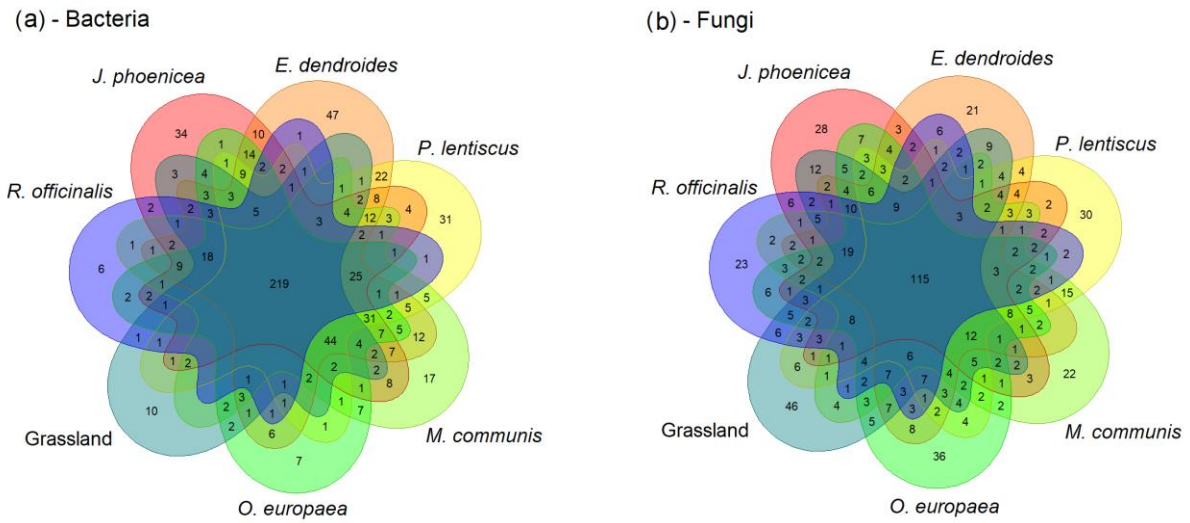

Fig 4. Venn diagram for bacterial (A) and fungal (B) communities show the numbers of ASVs (97% sequence identity) that were shared or not shared by the studied plant individuals depending on overlaps.

At the lowest taxonomic level (Fig. 6), clustering of the species, based on the 50 most abundant fungal ASVs, using the Bray-Curtis similarity index showed clear representation of variation among the fungal community across different shrubs and grassland (p = 0.001; Table S2). This clustering resulted in 7 separated clusters. The first cluster (A) represents *M. communis*, which was characterized by a group of genera including *Penicillium*, *Gibberella*, *Helicoma*, and *Pilidium*. The second cluster (B) where *J. phoenicea* was characterized with a group of ASVs that included *Stemphylium*, *Agaricomycetes*, *Mortierella* and other *Ascomycota* genera. The cluster C included *R. officinalis* and characterized by the genera *Didymella*, *Sarocladium*, *Phaeosphaeria*, *Xylariales* and *Russoella*, all belonging to the phylum *Ascomycota*. Moreover, cluster D included grassland and characterized mainly by *Claviceps* genus belonging to the *Ascomycota*. Cluster E, of *O. europaea*, was characterized by a group including *Pyrenochaeta* and *Verrucocladosporium*. Finally, cluster G that includes *P. lentiscus* was characterized by *Pyronemataceae*, *Arthrinium*, and *Didymellaceae*.

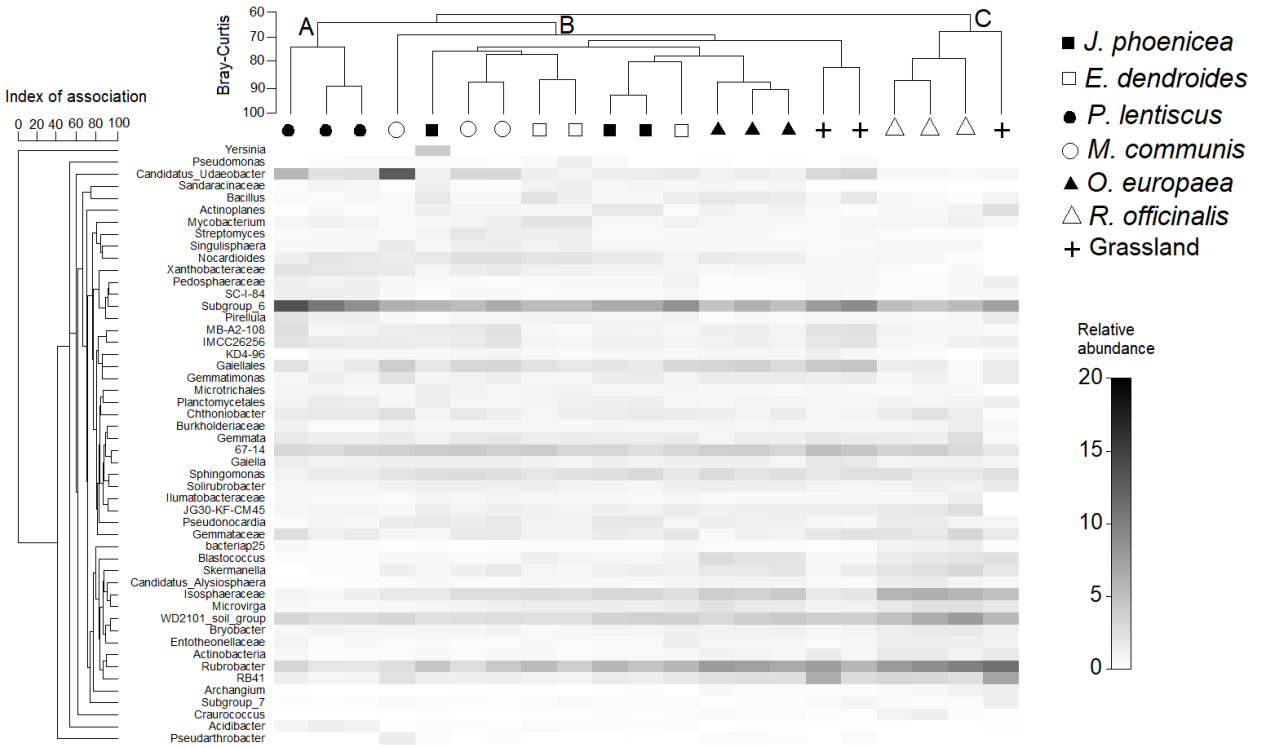

Fig 5. Heatmap showing relative abundance of the 50 most frequent Amplicon Sequence Variants (ASVs) in the bacterial community in the soil of each plant across the shrubland and grassland. The hierarchical clustering of samples is done by dissimilarity Bray and Curtis measure whereas the grouping of variables is based on Whittaker's association index. Capital letters refer to three main clusters discussed in the text.

As for the sample ordination based on the fungal community, *P. lentiscus* had the highest positive correlation with Ca (Fig. S4b). However, Fe was responsible for the ordination of grassland and *R. officinalis* with the highest positive correlation observed, while the large negative correlation between Fe and the fungal community of *M. communis* was responsible for their ordination. In addition, P, Zn and OC were negatively correlated with grassland, *R. officinalis* and *O. europaea* and positively correlated with *M. communis*. Finally, the ordination based on the fungal community of *J. phoenicea* was due to its strong negative correlation with Ca.

As revealed by FUNGuild analysis (Fig. S5), the distribution patterns of dominant ecological guild functions were more similar between *P. lentiscus*, *R. officinalis* and the grassland. However, each plant species was found to host one or several guilds whether exclusively or in more abundance compared to the others. For example, algal parasites are present only in the soil under *E. dendroides* and *P. lentiscus*; however, bryophyte parasites are

more abundant under the soil of *E. dendroides*, while they are almost absent in the soil of *R. officinalis* and the grassland. In addition, ectomycorrhizal fungi were present under all soils with low abundance except in grasslands where they were almost absent.

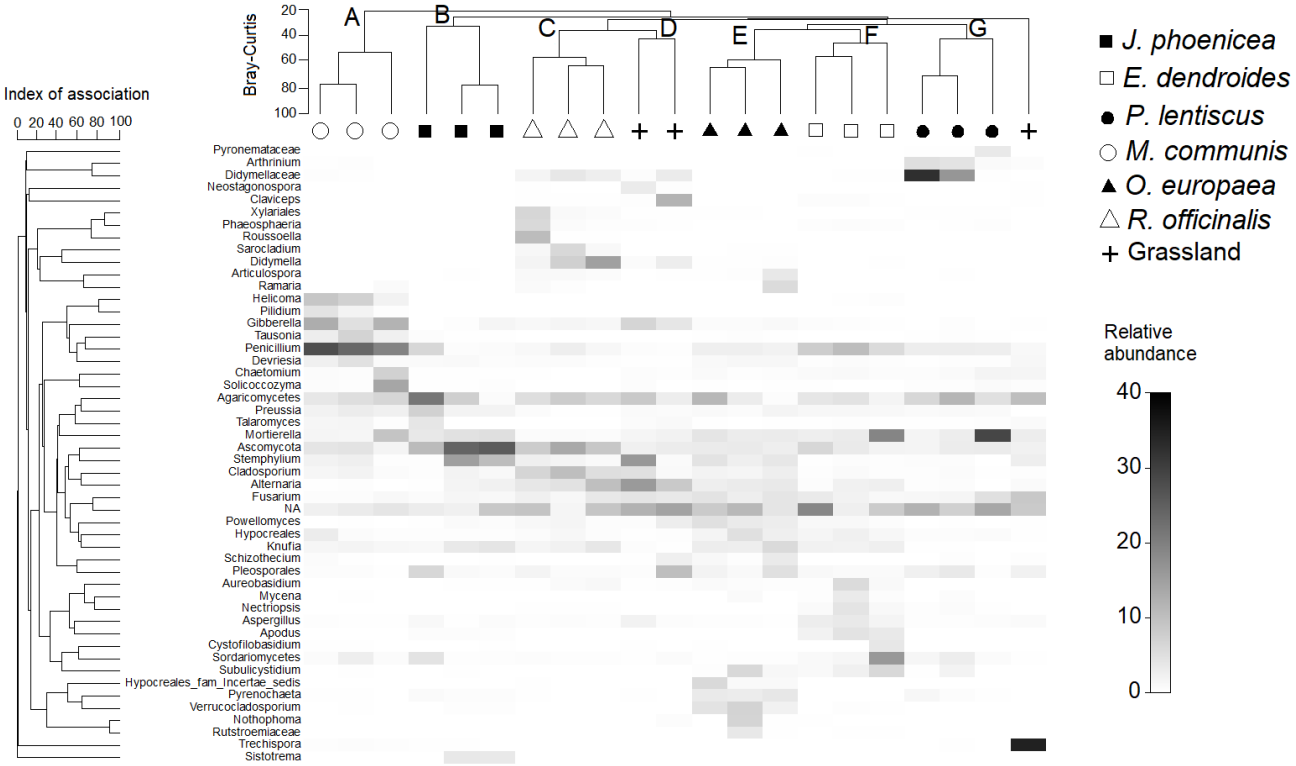

Fig 6. Heatmap showing relative abundance of the 50 most frequent Amplicon Sequence Variants (ASVs) in the fungal community in the soil of each plant across the shrubland and grassland. The hierarchical clustering of samples is done by dissimilarity Bray and Curtis measure whereas the grouping of variables is based on Whittaker's association index. Capital letters indicate the seven main clusters of the different plant communities discussed in the text.

4.4.5 Structural equation modeling

Hypothetical relationships between litter chemistry, soil chemical parameters and microbial diversity and composition were tested using structural equation modelling (SEM) across the shrubland (Fig. 7). This model showed that litter chemical parameters showed both positive and negative correlations with soil chemical properties. For example, lignin content of litter showed a negative relationship with soil P and organic carbon content with correlation coefficients of -0.738 and -0.350, respectively. In addition, N content of litter is positively related to soil pH, N content and soil organic carbon content with correlation coefficients of 0.718, 0.648 and 0.895, respectively, while it is strongly negatively correlated with soil N content with a coefficient of -0.999. On the other hand, this model showed that only pH

significantly negatively affected soil bacterial composition with a correlation coefficient of -0.977. On the other hand, fungal composition and diversity were affected by most of the soil parameters tested. Specifically, pH showed a negative correlation with both fungal diversity and fungal composition with coefficients of -0.958 and -0.937, respectively. In addition, organic carbon and P content showed a significant positive relationship with both fungal parameters, while N content showed a strong negative relationship with fungal diversity. Moreover, only cellulose content of litter showed a direct relationship with microbial parameters as it showed a positive correlation with bacterial and fungal composition and fungal diversity.

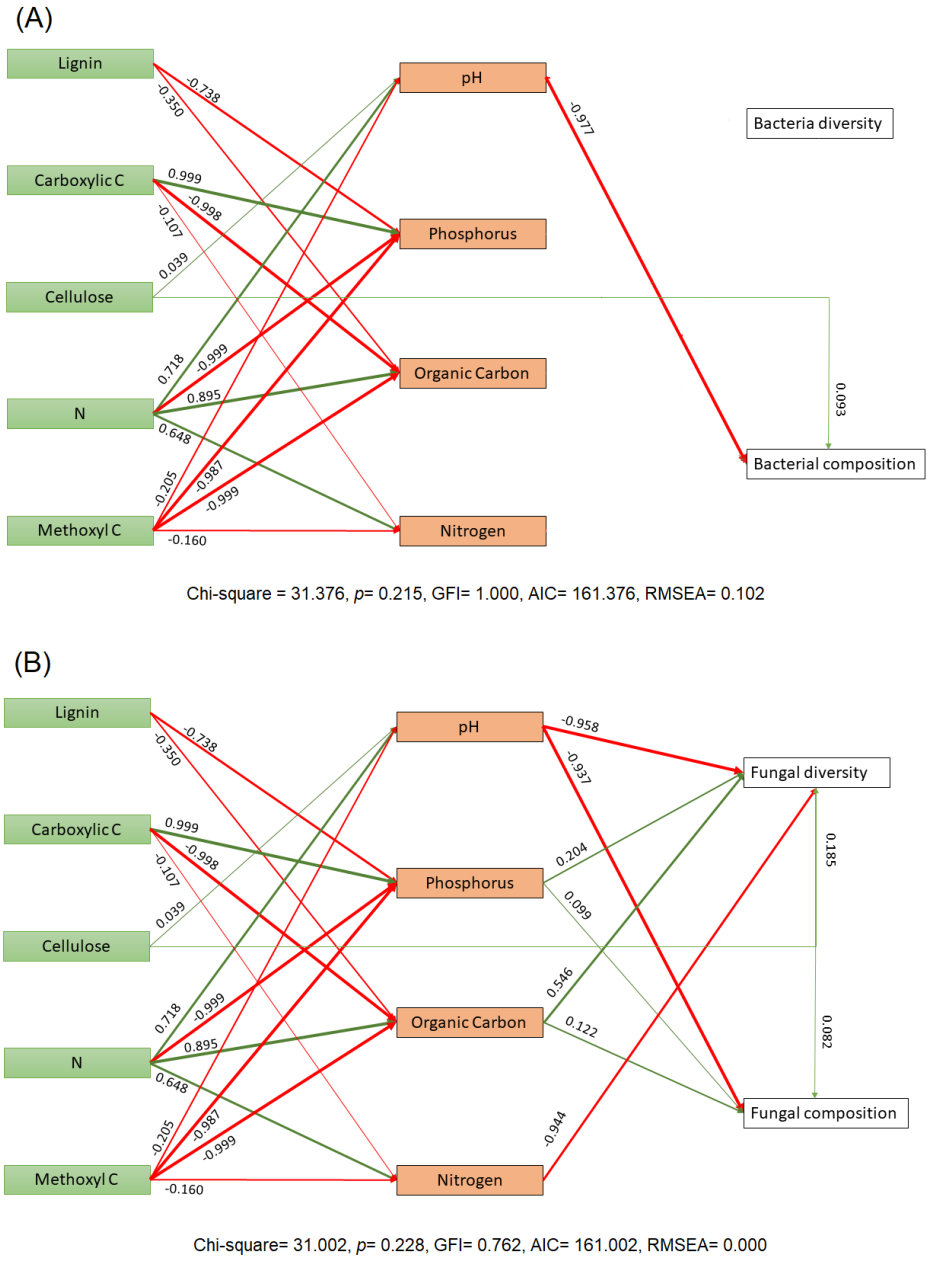

Fig 7. Structural equation model (SEM) shows influential factors of litter properties on the soil chemistry and on bacterial (A) and fungal (B) diversity and composition. Green and red arrows indicate

positive and negative relationships, respectively. Solid and dotted lines represent significant and insignificant differences, respectively. The thickness of the arrow represents the strength of the relationship. The low chi-square, nonsignificant probability level ($p > 0.05$), high goodness-of-fit index (GFI), and low root-mean-square errors of approximation (RMSEA) listed below the SEMs indicate that our data matches the hypothetical models.

4.5 Discussion

4.5.1 Grassland matrix

Our proximate and ^{13}C CPMAS NMR analyses showed that litter of *Ampelodesmos*, the dominant perennial grass in the monitored grassland, contained the highest levels of cellulose, di-O-alkyl-C, and O-alkyl-C compounds, while the levels of lignin, N, carboxyl-C, O-substituted aromatic C, methoxyl-C, and alkyl-C were the lowest. This litter type decomposes relatively quickly on the ground (Bonanomi et al., 2019), but is also highly flammable and most of it remains in the plant tussock, a condition that promotes the occurrence of fire events in this grassland (Incerti et al., 2013). Since litter input could affect soil ecological processes, including soil C and N cycling via litter decomposition, we observed that soil under *Ampelodesmos* had correspondingly lower levels of OC, total nitrogen, and P, while soil Fe content was highest. This is probably due to the occurrence of recurred summer fires, which allow low accumulation of litter and OC in the soil profile. In addition, we found low bacterial and fungal taxonomic diversity in grassland soil. These results could be associated with specific litter chemistry, i.e. low C/N ratio and labile C forms, and the low litterfall caused by the tussock structure that maintains standing litter until a fire event.

Overall, we found high abundance of *Acidobacteria* and *Actinobacteria* at the phylum level, whereas the abundance of *Proteobacteria* was low compared to the soil under shrub species. *Proteobacteria* are generally considered more copiotrophic, while *Acidobacteria* are considered oligotrophic in soil. Fierer et al., (2007) described *Acidobacteria* as oligotrophs that prefer poor soils with lower carbon availability. Therefore, their high proportion in grassland could be explained by the low C and N contents in the soil. On the other hand, in grassland, we found the higher abundance of *Basidiomycota* and the lower of *Ascomycota* compared to the soil under shrub species. Although the oligotrophic-copiotrophic theory has been intensively discussed in the field of bacteria, it is less frequently applied to soil fungal taxa (Yao et al. 2017). *Basidiomycota* fungi are known to be oligotrophic and generally capable to colonize and exploit recalcitrant carbon sources like lignin, suberin and other plant-derived compounds

(Lindahl et al., 2007; McGuire et al., 2013). Instead, saprotrophic fungi that belong to *Ascomycota* generally exhibit copiotrophic tendencies as they utilize freshly fallen plant litter rich in labile carbon forms (Lindahl et al., 2007; Crowther et al., 2012; Banonami et al. 2019). At low taxonomic level, we recorded a high abundance of the genus *Claviceps* in the grassland soil, whereas it was almost absent under all shrub species. This result suggests a rather specific association between *Ampelodesmos* and *Claviceps*, which are known for the ergot disease infecting ~200 species of wild and cultivated grasses (Boestfleisch et al., 2015).

4.5.2 Are shrubs' signatures specific?

We hypothesized that the diversity and composition of the microbial community would be altered by each shrub species, possibly due to the higher amount and/or diversity of litter trapped under the shrub canopy, which would enter the soil C and N cycles (Hooper et al., 2000). Our ^{13}C CPMAS NMR analysis shows high diversity within the litter characteristics of the shrubs studied compared to the grassland. We found that the litter of the evergreen, scherofillus *R. officinalis* had a high content of lignin and N while it had the lowest cellulose content. On the other hand, *P. lentiscus* had the lowest N content. Moreover, *O. europaea* had the highest value of methoxyl-C, while *M. communis* litter had the lowest lignin content. *E. dendroides*, a deciduous species that sheds its leaves in summer to avoid drought period, had high N content associated with high carboxyl-C and methoxyl-C content. As a result, *E. dendroides* accumulated little OC in the soil compared to other shrubs such as *P. lentiscus* and *O. europaea*. Moreover, our SEM analysis showed that litter properties exhibited significant direct effects on soil chemical properties and that these soil chemical parameters have shown both direct and indirect effects on bacterial composition and fungal composition and diversity. Previous studies suggest that litter decomposition in soil can alter microbial biomass, composition and community structure by increasing substrate variability and diversity of chemical compounds and that this can vary depending on litter quality (Chapman et al., 2013). Accordingly, the evergreens *P. lentiscus*, *O. europaea* and *M. communis* have high OC levels and low N levels in their soils, while the coniferous *J. phoenicea* and the scherofillus *R. officinalis* enclose low OC and N levels. Our results, at microbiota scale, showed that bacterial diversity was significantly higher under *J. phoenicea* and *E. dendroides* than under *R. officinalis*, while fungal diversity was significantly higher under *O. europaea* than under *M. communis*. Collins et al., (2020) found that shrub encroachment did not assign a "global signature" but was associated with increased, decreased, or no change in alpha microbial diversity when compared to soils from nearby herbaceous plant communities. Instead, our data

indicate, based on the PERMANOVA test, that the microbiota signature among coexisting shrub species is species-specific.

Previous work has shown that shrub encroachment increases oxygenation and nutrient content in surface soil (Bragazza et al., 2015), suggesting that SE may influence the distribution of bacterial life strategies in the soil, i.e. enrichment of copiotrophic and depletion of oligotrophic bacteria. The increase in *Proteobacteria*, a copiotrophic pylum, in shrub soils is consistent with the findings of Wallenstein et al., (2007), who found an increase in *Proteobacteria* in Arctic nutrient-rich shrub soils. Moreover, all shrubs harbored a significant amount of *Actinobacteria* in their soils. In particular, the *Streptomyces* genus was more abundant under *M. communis* compared to the other shrubs and the grassland. In this context, Qiao et al., (2017) studied microbial communities in nutrient-rich soils and found that *Actinobacteria* were more abundant than other microbes. It is now widely accepted that the establishment of bacterial communities in soils is not random but is controlled by specific compositional rules including plant species (Edwards et al., 2015). Although different plant groups do not necessarily differ in the size of their bacterial populations, aromatic plants, i.e. *J. phoenicea* and *R. officinalis*, are generally more colonized than other species (Yadav et al., 2005). A wide range of secondary metabolites, including nitrogen- and sulfur-containing compounds, benzoids, phenylpropanoids, and terpenes, are emitted as volatile organic compounds from below- and above-ground aromatic plant tissues, where they act over both short and long distances on a variety of soil microbial communities (Junker & Tholl, 2013). Interestingly, we found high abundance of free-living N-fixing bacteria, including the genera *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Neorhizobium*, under *E. dendroides* and *P. lentiscus*, while it was lower under *R. officinalis*. Surprisingly, the abundance of these free-living N-fixing bacteria was positively correlated with soil pH, phosphorus content and cations, while it was negatively correlated with soil Fe content, which could partially explain their distribution. Our results also showed that *R. officinalis* had the highest Fe content, which assigns for it an intermediate-like level to grassland, while *E. dendroides* had a very low soil Fe content. Fe plays a fundamental role in all isozymes of nitrogenase, the ubiquitous enzyme involved in biological N fixation (Raymond, 2003). This contradicts the negative correlation between Fe and the abundance of N-fixing bacteria under *R. officinalis* and grassland soils, which contain high amounts of mineral Fe. In this regard, it is possible that Fe is immobilized in the grassland soil and thus become unavailable to microbes, as well as to plants.

The abundance of fungal phyla showed a marked variation among shrub species. Our findings are in line with earlier studies showing that the *Ascomycota*, which are early colonisers

of litter and the major decomposers, are litter type-specific (Šťursová et al., 2020) and thus highly abundant under shrubs. The phylum *Basidiomycota* is generally better equipped for lignin degradation (Lundell et al., 2010); our results confirm that the highest amount of lignin was found in the litter of *R. officinalis*, so the abundance of *Basidiomycota* is positively correlated with the lignin content in the litter of shrub species. Moreover, *Mortierellomycota* are known to be saprobic and ubiquitous, and several studies show that they have the ability to solubilize P and are associated with increased yields and establishing symbioses with plants (Grządziel et al., 2019). Our results showed that *M. communis* was characterised by a group of fungi, composed mainly of *Penicillium*, which are among the common producers of secondary metabolites in soil and have played a role in the early stage of litter decomposition (Frisvad & Samson, 2004). Park et al., (2020) found that most *Penicillium* species from soil are highly selective and unique to each plant.

E. dendroides was characterised by the presence of a significant amount of weak saprophytes, including *Aspergillus*, *Alternaria* and *Cladosporium*, while *R. officinalis* exclusively hosted the genus *Didymella*, which are opportunistic parasitic microorganisms that often exploit special conditions to colonise on plants and occasionally cause severe damage (Blancard, 2012). *E. dendroides* also exclusively harbours the genus *Aureobasidium*, a typical phyllospheric endophyte that is mainly found in fresh litter and rapidly disappear upon decomposition (Bonanomi et al., 2019). According to previous culture-based studies conducted on different tree species, the persistence of *Aureobasidium* in decomposed litter is unusual due to its limited competitive ability (Voříšková & Baldrian, 2013). Our finding, that different functional groups of litter stimulated different fungal taxa, suggests that fungi have a preference litter types, probably because the ability to degrade specific organic compounds varies among taxa (van der Wal et al., 2013).

In our study, no shrubs form symbiosis with ectomycorrhiza, except *J. phoenicea* (Mejstrik & Cudlin, 1983), therefore, their presence under shrub species is in the form of free-living spores. The fact of the presence of these mycorrhizal fungi confirms the formation of islands of fertility under the shrubs, indicating that the soil is ready for vegetation succession.

Our results suggest that the composition of the fungal microbiota converges in part by the types of litter functional group enclosed by each shrub species (Reinhart & Callaway, 2006). This could be due to differences in chemical composition between litter types, with plant functional groups often playing an important role in explaining differences in litter chemical traits (Diaz et al., 2004). In contrast to our results for fungal communities, we did not find strong shifts in the community composition of bacteria. These results were further confirmed

by our SEM analysis, which showed that soil chemical parameters had rather insignificant indirect effects on bacterial composition and diversity, with the exception of pH, which showed a negative direct effect on bacterial composition. That the effects were particularly pronounced for fungal communities may not be surprising, considering that fungi play a key role in the degradation of more recalcitrant organic compounds (van der Wal et al., 2013). It could be that fungi are more specialised to certain litter types, while bacteria use simpler carbon compounds from litter and fungal degradation products and are therefore less responsive to different litter types.

4.6 Conclusion

Our study was able to verify the effect of "islands of fertility" caused by the shrub encroachment over the grassland matrix. Indeed, soils under shrub species enclosed higher levels of OC, total nitrogen and P. Moreover, our study discovered that under the same Mediterranean climate and limestone rock, coexisting shrubs generate specific signatures of bacterial and fungal microbiota in the soil. However, fungal community composition was the best indicator of the impact of different shrub species on the soil. We found that shrubs differed not only from the matrix of the *A. mauritanicus* grassland, but also between shrubs belonging to different functional groups, i.e. deciduous, coniferous evergreen and sclerophyllous evergreen. Bacterial diversity was significantly higher under deciduous *E. dendroides* than under coniferous *R. officinalis*. Moreover, fungal diversity was different even among the two evergreen *O. europaea* and *M. communis*. Differences in litter chemistry likely play a key role in changing soil chemistry and, in a cascade, shaping the soil microbiota. However, according to the results of our SEM analysis, the fungal community was more dependent on soil chemical characteristics than the bacteria. The observation that shrub signatures are specific is not trivial and highlights the limitations of the common approach that generalises the effect of shrubs compared to open vegetation habitats. Moreover, the presence of a specific microbiota under shrub species is likely the result of a species-specific plant-soil feedback that probably took decades to develop. Indeed, this raises new questions about the functional consequences and direction of such feedback for seedling recruitment and species coexistence, as well as for litter decomposition in the context of home field advantage's framework.

5 Chapter 5: What drives distance dependency recruitment of *Euphorbia dendroides* in a Mediterranean shrubland: Microclimate, soil chemistry or microbiota?

5.1 Abstract

Species coexistence in plant communities is enhanced by disturbance, pathogens, and predators. Pathogen activity can cause density and distance effects leading to Janzen-Connell (JC) distribution. The JC hypothesis states that there is a low probability that a dead tree will be replaced by a conspecific, which promotes species coexistence and prevents monodominance. Early studies identified insects and predatory mammals as major causes of JC. However, later studies also identified the activity of soil pathogens and harmful soil microbes as possible causes. In this study, we first investigated and quantified whether JC occurs for *Euphorbia dendroides* in a mixed shrubland and quantified recruitment under heterospecific plants as well. We then investigated the ecological causes of the observed pattern. Specifically, we investigated whether soil chemistry and/or soil microbiota, characterized for both bacterial and fungal communities by next-generation sequencing, explained the observed pattern of seedling recruitment. We also investigated differences in microclimate among shrub species by measuring air temperature and light at different times of the year. The results showed that *Euphorbia* seedlings are almost absent under the canopy of conspecifics, but are more abundant at the edge of the canopy and increase thereafter, which fits perfectly with the JC model. Interestingly, we also found a high density of *Euphorbia* seedlings under heterospecific canopies, i.e. *Olea* and *Juniperus*, but a very low density under other heterospecific canopies, e.g. *Rosmarinus* and *Pistacia*. Remarkably, all of the few seedlings found under *Euphorbia* conspecific canopies were less than 2 years old, while those found outside the canopy reached an age of more than 10 years. In our study, the age of *Euphorbia* recruits peaks at a distance of 1 to 2 m from the adult tree and decreases with increasing distance. Moreover, our results showed that the observed JC pattern of *Euphorbia* could not be explained by the accumulation of species-specific pathogens in the soil or by changes in soil chemical and microbial properties. Moreover, the observed JC pattern cannot be explained by the air microclimate under the canopy of conspecifics and heterospecifics either. For this reason, further analyses are needed to better explore the mechanisms behind the observed JC pattern, probably exploiting the autotoxicity theory, which states that during the decomposition of conspecific litter, some allelochemicals, including self-DNA, are released in the soil and cause specific auto-inhibition.

Keywords: Janzen-Connell distribution, soil pathogens, conspecifics, *Euphorbia dendroides*, next-generation sequencing, seedling recruitment.

5.2 Introduction

Species coexistence in plant communities is allowed by disturbance, pathogens, and predators (Kim & Ohr, 2019). Negative distance-dependent mortality could lead to the so-called Janzen-Connell (JC) distribution (Janzen, 1970; Connell, 1971). The JC hypothesis states that seeds are most likely to disperse at sites near their parent trees, where they are also most likely to be attacked by host-specific enemies such as insects and soil-borne pathogens. In contrast, seeds and seedlings dispersed further from the parent tree are more likely to survive as they escape natural enemies (Janzen, 1970). The JC hypothesis predicts that there is a low probability that a tree will be replaced by a conspecific, a process that promotes species coexistence and prevents monodominance (Bagchi et al. 2010; Murphy et al. 2017). Janzen (1970) summarized his hypothesis in a simple graphical model consisting of two curves. The first describes seed dispersal around a focal tree and the second gives the probability of seed survival as a function of distance from the focal tree. Accordingly, despite the high density of dispersed seeds, recruitment density near adult trees is relatively low due to the strong effect of seed predators; it increases to a peak at a certain distance and then decreases at greater distances due to the low density of seeds away from the source. The formation of such exclusion zone by conspecifics gives rare species an advantage and promotes species coexistence and community diversity maintenance (Jia et al. 2020).

Early studies identified insects and mammalian predators as major causes of JC (Clark & Clark, 1984; Hammond & Brown, 1998). For example, Janzen (1975) reported that two species of bruchine beetles in Costa Rica are host-specific to the seeds of *Guazuma ulmifolia*, with one species attacking the seeds on the tree before dispersal and the other attacking only the mature seeds after they have fallen to the ground. However, later studies also identified soil-borne pathogen activity and harmful soil microbes as possible causes of JC distribution (Bagchi et al. 2010; Fricke et al. 2014). Packer & Clay (2000) reported that *Prunus serotina* seedlings have high mortality in soil collected beneath conspecific adults, but low mortality in soil collected beneath heterospecific adults. Seedling mortality was attributed to the pathogenic oomycete *Pythium* spp. isolated from soil and the roots of dying seedlings. Despite these particular cases, only few studies have been able to identify the species of natural enemies involved in this process, and most studies only captured the recruitment pattern without investigating the underlying mechanisms (Basset et al. 2019).

The JC hypothesis was originally proposed to explain the high diversity of tropical forests. However, in contrast to the very large number of studies for tropical forests (review in

Wright, 2002; Comita et al. 2014; Song et al. 2021), few studies have investigated the occurrence of JC distribution in temperate zones (Packer & Clay, 2000) and in Mediterranean forests (Nathan et al. 2000) or shrublands (Bonanomi et al. 2008; Teste & Laliberté, 2021). Compared to continuous forests, shrublands have more discontinuous woody plant cover (Li & Strahler, 1988), leading to heterogeneity and patchiness in terms of abiotic, microclimatic conditions and resource availability (Facelli & Temby, 2002). The microclimate in Mediterranean shrublands differs largely in the microsites under the canopy of shrubs and interspace covered by herbaceous vegetation, which could have significant implications for plant recruitment (Callaway, 2007). The canopy reduces direct solar radiation (Maestre et al. 2001) and indirectly affects air and soil temperature (Magid et al. 1999). For example, in the northern Chihuahuan Desert, He et al. (2015) found that the average minimum winter temperature under shrub canopy was about 2-4 °C higher than under grassland cover. In Mediterranean climates, Stinca et al. (2015) reported that the canopy of *Genista aetnensis* buffers extremely high temperatures at the air and in the topsoil. As a result, shrub canopy could facilitate seedling establishment by buffering extreme temperatures thus acting as nurse plants (Castro et al. 2004).

Landscapes in a Mediterranean ecosystem are characterised by dispersed vegetation with higher soil fertility under plant canopies, leading to spatial heterogeneity of soil resources and favouring the formation of "fertile islands" (Ridolfi et al. 2008). The formation of fertility islands depends on the amount of fallen litter, chemical properties, decay rate and associated accumulation of soil organic carbon. The differences in leaf chemistry and decomposition rates among tree species likely result in a different spatial pattern of soil properties associated with the plant canopy. It is also suggested that differences in plant canopies and associated soil chemistry lead to differences in soil microbiome (Kushwaha et al. 2021). For example, Idbella et al. (2022) recently found, in a Mediterranean shrubland, fertility islands under shrubs with associated microbiome signature related to different plant traits i.e. deciduous, evergreen, coniferous and sclerophyllous. More generally, plant pathogens, epiphytes, endophytes, saprotrophs, and mycorrhizae are associated with canopy soils at different abundances depending on the woody perennial species. Soil microbes have a major impact on plant survival, population dynamics, and species distribution (Klironomos, 2002; Schnitzer et al. 2011). Some microbes have a negative impact on plants (Bagchi et al. 2010), while others have a positive influence, e.g. microbial mutualists that can help with soil nutrient uptake or defence against herbivores (van der Heijden et al. 2008). For example, the main role of arbuscular mycorrhizal fungi (AMF) is thought to be facilitating phosphorus uptake by plants, especially

in highly P-limited soils (Howeler et al. 1982). However, there is ample evidence that AMF also have an important influence on plant-pathogen interactions and are able to reduce pathogenic infections (Klironomos, 2002; Herre et al. 2007). Some studies have found evidence that the beneficial effects of AMF on seedling should also decrease with distance from the parent tree (Bever, 2002; Bidartondo et al. 2002), thus leading to the so-called reverse JC pattern (Zahra et al. 2021).

Early field observations (Bonanomi G., personal observation) suggest that *Euphorbia dendroides* (hereafter *Euphorbia*), a deciduous shrub, exhibits a recruitment pattern consistent with the JC distribution. Here, we first quantified whether JC distribution recruitment effectively occurs. Moreover, because *Euphorbia* coexists in a species-rich shrubland with five woody species, namely *Pistacia lentiscus* L., *Juniperus phoenicea* L., *Myrtus communis* L., *Rosmarinus officinalis* L., and *Olea europaea* L., we also quantified recruitment under heterospecific shrubs (species are indicate hereafter with genus name). We then investigated the ecological causes of the observed pattern. Specifically, we examined whether soil chemistry and/or soil microbiota, characterized for both bacterial and fungal communities by next-generation sequencing, explained the observed pattern of seedling recruitment. We also explored differences in microclimate among shrub species by monitoring air temperature and light availability at different times of the year. Since the shrubs we studied belong to different functional groups, we expect some divergence in terms of the microclimatic parameters measured. Specifically, this work tested the following hypotheses: i. *Euphorbia* recruitment is consistent with the JC distribution model; ii. Density of *Euphorbia* recruitment is positively correlated with soil fertility; iii. Density of *Euphorbia* increases with light and with temperature buffering effect; iv. Recruitment density of *Euphorbia* is positively correlated with beneficial soil microbes' i.e. mycorrhizal fungi, and negatively with soil-borne pathogens.

5.3 Material & Methods

5.3.1 Study site description

The study was conducted in Cape Palinuro shrubland site (40°01'35 "N 15°16'30 "E), located in south-western Italy, about 64 km southwest of the city of Salerno (Fig. S1). This area is located in a Mediterranean climate characterised by mild winters and hot and dry summers. The vegetation was open grassland dominated by *Ampelodesmos mauritanicus* L. tussocks (average height was 120-150 cm) intermixed with herbaceous vegetation and scattered shrubs and trees (Fig. 1A). The vegetation is adapted to dry summers and is fragrant and oily, making

it susceptible to fire. The elevation of the study area is 185 m a.s.l. The average annual temperature is 16.7 °C, with monthly temperatures ranging between maxima of 24.5 °C (August) and 10.2 °C (January). Average annual precipitation is 789.8 mm with the rainy season extends from winter through spring and autumn, with a marked dry season in summer. The site is characterised by limestone rocks overlying clay soils with abundant rock outcrops.

5.3.2 Study species description

Euphorbia is a deciduous, single-stemmed, semi-succulent shrub up to 2m tall, common in Mediterranean areas along the coasts and especially on rocks, cliffs and arid calcareous soils (Traveset & Sáez, 1997). *Euphorbia* forms spherical bushes that flower in February-March (Fig. 1A, B). Peak flowering is usually in mid-March and lasts 2 to 3 weeks (Fig. 1D; Traveset, 1995). Flowering begins no earlier than December. The first seeds mature in late April, and the foliage, which is often red, finally falls off in May or June. The seeds are brown, roundish, smooth, relatively large (the weight of 1000 seeds is 6.2g) and can be propelled several metres away from the plant by gravity. The seeds are usually short-lived, but can be viable for about five years. After fruiting, the species exhibits summer-deciduous foliage behaviour that coincides with the summer dry season and sheds its leaves until the autumn (Fig. 1C). In addition, *Euphorbia* secretes an irritant latex when cut, which may serve as a defence for the plant against insect predators and pathogens (Hua et al., 2017).

Pistacia is an evergreen perennial shrub up to 5m tall with dense foliage. It retains its foliage throughout the year and grows slowly. *Pistacia* species are widely distributed in the Mediterranean region and areas around the Mediterranean Sea (Ierapetritis, 2010). It thrives on a variety of soil types and is able to tolerate and accumulate salt; it also tolerates long periods of high sunlight and high temperatures (Landau et al., 2014). *Pistacia* leaves have been found to contain high levels of flavonoids, phenolic acids and tannins (Remila et al., 2015). *Juniperus*, on the other hand, is a large shrub that grows up to 8m tall, with a trunk up to 2m in diameter and a round or irregular crown. *Juniperus* prefers a hot, arid climate with plenty of light and grows in rocky or sandy soil. Its preferred soil is calcareous and has a moderately alkaline pH. *Myrtus*, however, is an evergreen, aromatic shrub that grows up to 5m tall. The leaves are up to 5cm long and contain a strongly fragrant essential oil. It is well adapted to water stress conditions and can be used to revegetate arid and degraded areas. Furthermore, *Rosmarinus* is an evergreen shrub with leaves that resemble hemlock needles. It is a dense, aromatic plant with bright green leaves and is native to the Mediterranean region, but is reasonably hardy in cool climates. It can withstand periods of drought and will survive severe water shortages for

long periods. In some parts of the world, it is considered a potentially invasive species. The seeds are often difficult to start, with a low germination rate and relatively slow growth, but the plant can live up to 30 years. Finally, *Olea* is an evergreen tree native to the Mediterranean region. It usually grows up to 9m tall and has a round crown. *Olea* is drought, disease and fire resistant. Its root system is robust and capable of regenerating the tree even if the aboveground structure is destroyed. It shows a marked preference for calcareous soils and does best on limestone slopes and cliffs and in coastal climates. It will grow in any light soil, even clay, if it is well drained. Olea species like hot weather and sunny locations without shade, while temperatures below -10°C can damage even a mature tree.

Fig. 1. A: Image of the Shrubland site of Capo Palinuro located in Southern Italy in Campania region. B: Image of the studied *Euphorbia dendroides* adult shrub. Image of a recruitment during summer (C) without the leaves and during spring with leaves (D).

5.3.3 Assessment of *Euphorbia* recruitments

To assess the recruitment distribution pattern of *Euphorbia* within the shrubland, 20 mature shrubs were selected based on their size. Trunk diameter, height, and crown diameter were

recorded for each of the twenty mature shrubs. We then counted all seedlings that were under the parent shrub and in consecutive torus outside the canopy with a belt size of 1 meter within a radius of 4 meters (Fig. S2). For each seedling, we measured the height, age, and distance from the parent shrub. In order to determine the age of the seedlings in the different torus, the annual internodes of the main stem were counted for each seedling. Every year, the plant produces branches that end in a node, from which two or three branches are produced the following year with the same growth form of earlier ones. Therefore, by counting the nodes in a straight line of growth, it is possible to work out the age of the plant (Eichberger, 2003).

5.3.4 Microclimate under shrub canopies

The microclimate under shrubs as well in matrix grasslands was characterized for air temperatures. We use the AgriLogger sensor that belongs to a family of battery-powered sensors that can capture, store, and transmit measurements of two important environmental parameters-temperature and relative humidity (Idbella et al. 2020). Three sensors were manually placed one under each of the three replicates of each shrub studied, i.e., *Euphorbia*, *Pistacia*, *Juniperus*, *Myrtus*, *Rosmarinus*, and *Olea* (Fig. S2). Each sensor collected and stored one measurement per hour of air temperature. Data were collected in three different seasons: summer from 06 August to 30 September 2020, autumn from 1 October to 30 November, and winter from 1 December 2020 to 24 February 2021.

To obtain data about light availability in grassland and under shrub canopies, we used a light metre (LI - COR LI -250A) that measure photo-synthetically active radiation wavelengths between 400 and 700 nm. Measurements were made at 10 randomly selected individuals for each shrub species. Measurements were made during sunny, bright days in summer and in winter of 2020. Sensors were placed above the ground at a distance equivalent of the middle of the shrub trunk. Light intensity data under the shrub canopies were expressed as percentages compared to open grassland.

5.3.5 Soil sampling

Soil samples under each of the six shrubs and in the grassland were collected using a 5 cm diameter soil corer, at a depth of 15 cm after removal of above ground litter at four randomly selected points under each tree canopy and three replicate canopies for each shrub species, giving a total of 21 samples. The soil was then pooled and sieved (2 mm mesh), resulting in a single composite sample for each tree canopy replicate. Samples were stored in sterile plastic

bags and labelled. Prior to each sampling, the soil corer was thoroughly cleaned and sterilised to prevent contamination between samples. After collection, the samples were divided into two fractions: one fraction was stored at 4°C to study the chemical properties of the soil; the other fraction was stored at -20°C and used for molecular analysis.

5.3.6 Soil chemistry and microbiota

Soil chemistry and microbiota properties of the six shrub canopies and of the grassland were previously reported in Idbella et al. (2022). Here, these data were used as a reference dataset for correlating with *Euphorbia* recruitment. Briefly, soil samples were analysed for 15 parameters: soil electrical conductivity (EC) and pH were determined in soil-water suspensions at a ratio of 1:5 and 1:2.5 using a conductivity meter and a pH meter, respectively. Total nitrogen was determined by the Kjeldhal method, while phosphorus was determined by the molybdovanadate-phosphate method. Total organic carbon was assayed by the chromic acid titration method. Potassium, magnesium, iron, manganese, calcium, sodium, copper and zinc were determined by flame atomic absorption spectroscopy. Total limestone is determined by the weight method against a strong acid. (LANO: NF ISO 10693). Finally, chloride (Cl) content in soil was determined by the volumetric method.

Concerning soil microbiome, next-generation sequencing data on the bacterial and fungal microbiota were analysed using 16S rRNA and ITS gene sequences and are deposited in the NCBI Sequence Read Archive (SRA) under the Palinuro microbiome bio-project with accession number PRJNA744707. In our previous paper (Idbella et al. 2022), we found highly specific microbial signatures under each shrub species. Briefly, we found that soil bacterial species richness was significantly higher under the canopy of *Euphorbia* and significantly lower under *Rosmarinus* than in the open grassland. In addition, the Shannon bacterial index was significantly higher in the soil under the canopy of *Juniperus* and *Euphorbia* than under the canopy of *Rosmarinus* and in the grassland. On the other hand, no significant variation was observed in fungal species richness, while *Olea* had the highest Shannon diversity index of soil fungi. As far as fungal taxonomy is concerned, all the soils studied were dominated by the phylum *Ascomycota*. However, the highest proportion of the phylum *Basidiomycota* was found in the grassland soil, followed by *Juniperus* and *Euphorbia*. Moreover, *Pistacia* exclusively harboured the *Mortierellomycota* phylum, followed by *Euphorbia*, while it was almost absent under the *Rosmarinus* canopy.

5.3.7 Data analysis

Recruitment density under adult canopies was calculated by dividing the number of seedlings under each canopy by the area of the same canopy using the recorded crown diameter. In the case where the density within different torus is outside the conspecific canopy, the calculation was done by dividing the number of seedlings within the torus by the surface area of the torus. Boxplots were created using the ggplot2 package in R (version 3.3.2), and statistical differences were calculated using the ggsignif package, which calculates the significance of a difference between groups using one-way/multi-way ANOVA to determine if group means differ from each other, and then followed up with post-hoc multiple comparisons to make finer comparisons between different levels of the group.

Concerning the impact of microbiome on *Euphorbia* recruitment, the abundance matrices of ASVs were rarefied once to an equal number of reads per sample to reduce the effect of variation with respect to sequenced reads using the rrarefy function in R/ VEGAN (Oksanen et al., 2007). The Fungi: Bacteria ratio was calculated based on the proportion of fungal to bacterial metagenomic rRNA genes (Lange et al., 2014). Furthermore, we analysed functional group variation for the fungal community, identifying putative fungal functional groups as well as their trophic modes using FUNGuild (Nguyen et al., 2016). To generate density and ASVs distance matrices, the Bray-Curtis dissimilarity between each sample pair was calculated. The spatial autocorrelation as well as the correlation in the composition of the different guilds was calculated using the Mantel test (R/ VEGAN). Correlation networks incorporating communities containing bacteria and fungi were based on single ASVs and generated to assess correlations or potential interactions between single species and recruitment density. The pairwise correlations between the ASVs and density were calculated using the Spearman correlation in R (version 3.3.2 and Hmisc package 4.0–1). Based on the statistical analysis, only strong and significant (Spearman's $r>0.6$, $p>0.05$) correlations were considered. The network visualization was made using Gephi (version 0.9.2, Bastian & Jacomy, 2009). Each edge represents a robust and significant correlation and each node represents an ASV with the main central node that represents *Euphorbia* recruitment density.

Concerning microclimate, we tested for nonlinearity in the relationships between the average temperatures outside the studied tree canopies (i.e., inside the grassland) and the temperature offset throughout the season of record. Temperature offset is determined as the understory temperature minus the temperature outside the canopy: negative values thus reflect cooler temperatures below tree canopies while positive values reflect warmer understory temperatures (De Frenne et al. 2019). We used general additive mixed models (GAMMs) for

both stands including open-field temperatures and daytime (as sin and cos of hours) as fixed effects and seasons as random effects with the mgcv package (Wood, 2017). Moreover, we evaluated the extent to which open-field temperatures predicted variation in temperature among the studied canopies during the three different seasons, and the main differences among the six canopies were calculated in terms of canopy buffering capacity among the six canopies. We also determined how the season variable affected variation in canopy offset and how it interacted with macroclimate temperatures. First, we conducted a separate univariate LMM for the season variable as a fixed effect. Then, to test for interactions, we also conducted LMMs with two predictor variables each: macroclimate temperature and the season variable.

To determine the relationships between soil chemical properties and the calculated seedling's density, Pearson's correlation test was achieved with the Hmisc package in R software (Harrell & Dupont, 2018).

5.4 Results

5.4.1 Assessment of *Euphorbia* recruitments pattern

Average density of *Euphorbia* recruitments was lowest under canopy of conspecific with a density of 0.07 recruits/m^2, while the highest density was recorded within the first meter torus away from the conspecific plant with an average density of 0.33 recruits/m^2 (Fig. 2A). The lowest recruitment age of 2.2 years was recorded under the canopy of the conspecific plant, whilst the highest age was found in the first meter torus away from the conspecific (Fig. 2B).

Significant differences among recruits of *Euphorbia* was found under different shrub species (Fig. 3). The lowest average density was found under the canopy of the conspecific plant, while the highest average density was found under the canopy of *Olea* and *Juniperus* with intermediate values under other shrubs and in the grassland.

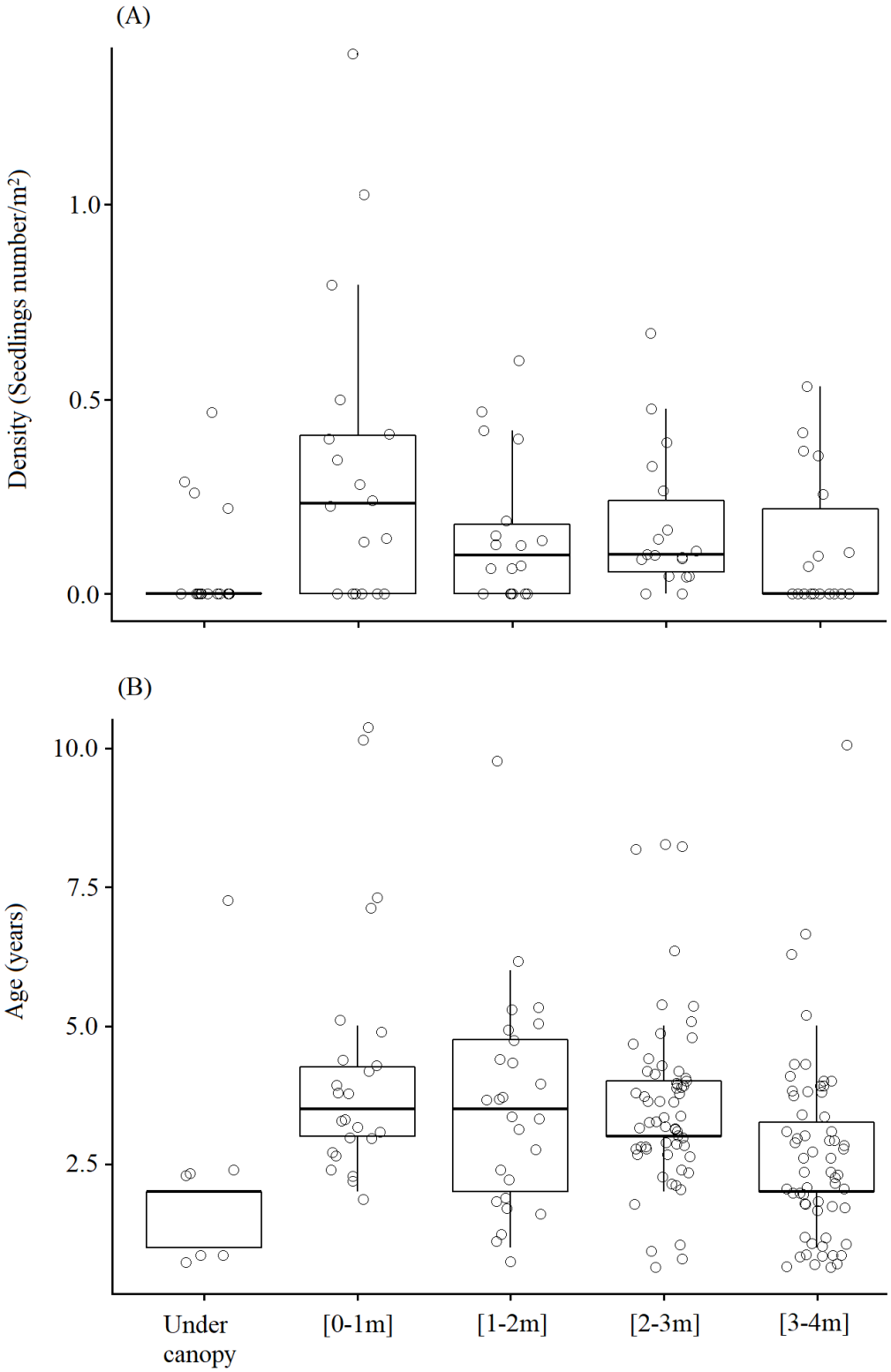

Fig. 2. Boxplot showing the density (A) and age (B) of *Euphorbia* recruitments under conspecific canopy and within different torus of 1 m away from the conspecific adult plant. The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median, whiskers above and below the boxplot indicate inter-quartile range.

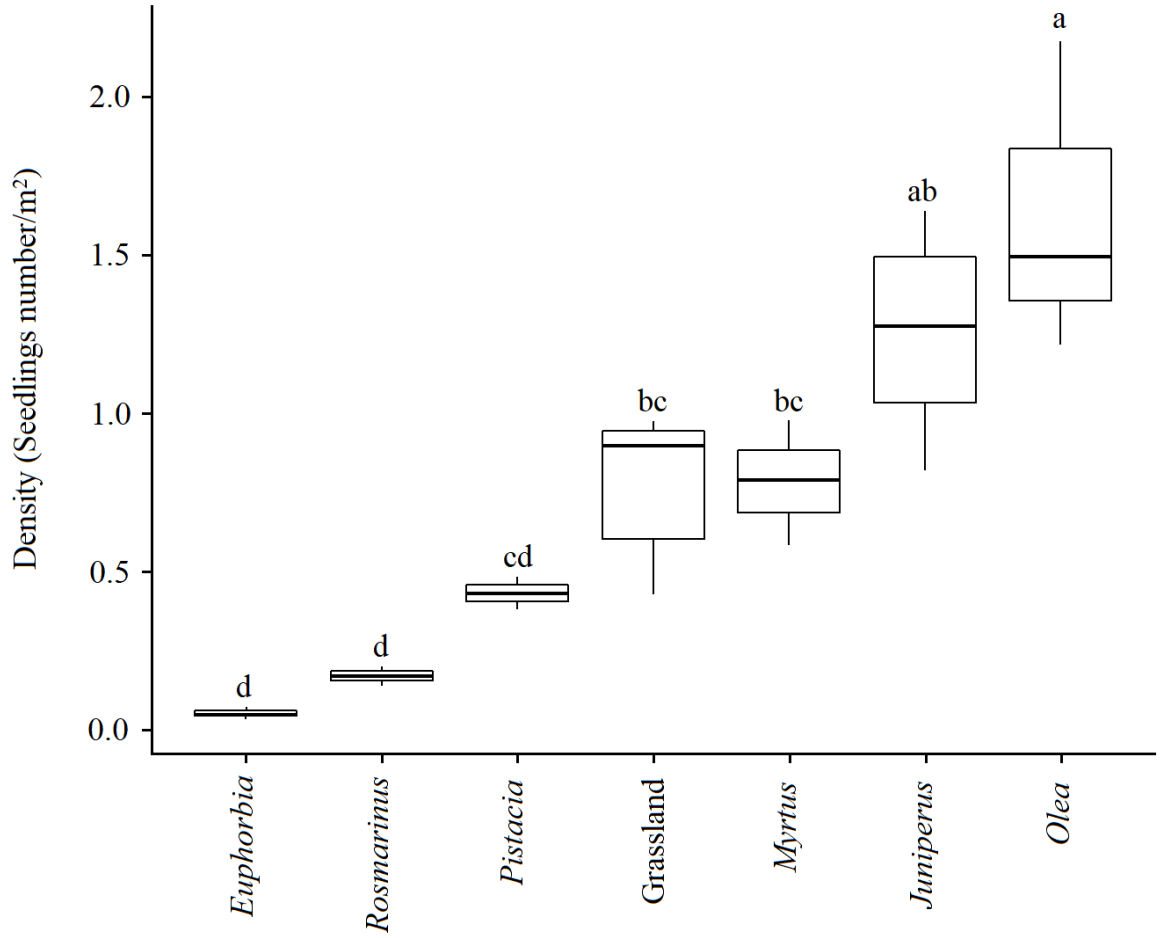

Fig. 3. Density of *Euphorbia* recruitments under the conspecific canopy and under heterospecific canopies including the grassland matrix. Different letters indicate significant differences according to Tukey *post hoc* test ($p<0.05$). The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median, whiskers above and below the boxplot indicate inter-quartile range.

5.4.2 Microclimate under shrub canopies

Mean temperatures under shrub canopies were on average 2.1°C, 1.1°C, 0.9°C, 0.8°C, 0.7°C, and 0.5°C cooler than temperatures outside the canopy for *Myrtus*, *Pistacia*, *Juniperus*, *Euphorbia*, *Rosmarinus*, and *Olea*, respectively. Overall maximum temperatures under the canopies were on average 6.2°C, 5.5°C, 5.4°C, 4.5°C, and 1.0°C cooler than the temperatures outside for *Juniperus*, *Myrtus*, *Olea*, *Pistacia*, and *Euphorbia*, respectively, while the overall maximum temperature under the canopy of *Rosmarinus* was 2.2°C warmer than the outside temperature. In addition, the overall minimum temperatures under the shrub canopies were 3.4°C, 1.3°C, 1.2°C, 0.7°C, 0.7°C, and 0.1°C cooler than the macroclimate outside the canopies for *Rosmarinus*, *Myrtus*, *Juniperus*, *Euphorbia*, *Pistacia*, and *Olea*, respectively (Fig. 4).

Under shrub canopies, temperature offset was generally negatively correlated with macroclimate temperature outside the canopy, especially under *Olea*, *Juniperus*, and *Myrtus*, whereas it was almost not significantly correlated for *Euphorbia*, *Rosmarinus*, and *Pistacia* (Fig. 4). Temperature offsets became more negative i.e., lower temperatures under canopies, with the increasing climatic temperature and more positive i.e., higher temperatures under canopies with the decreasing climatic temperature. In addition, the cooling of mean and maximum temperatures among all tree canopies studied was greatest in summer, while minimum temperatures among all shrub canopies were highest in winter (Fig. S3).

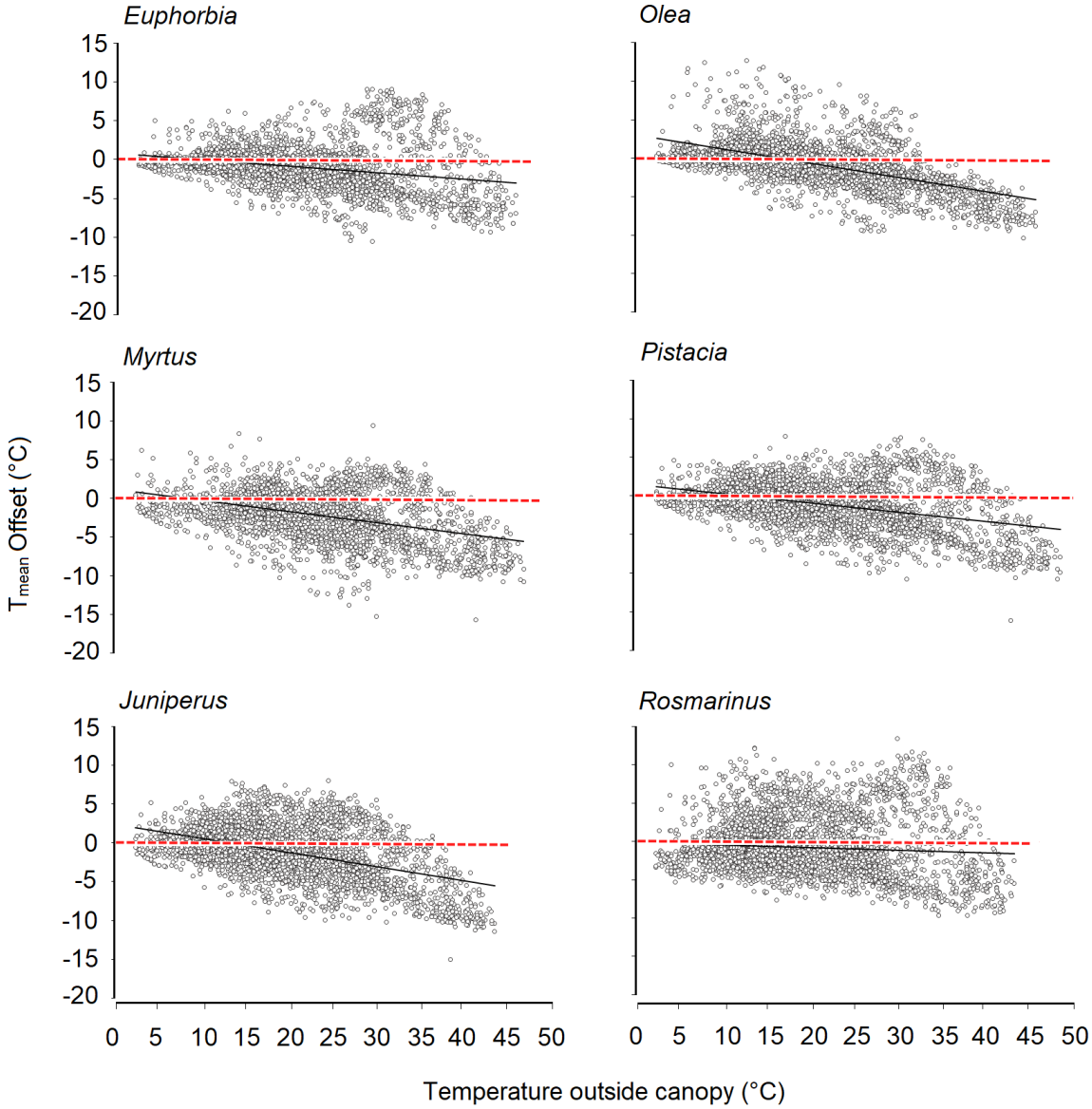

Fig. 4. Relationships between the average near-ground temperatures in open-field (set as reference) and below-canopy temperature offset during the measurement period from August to February under the canopy of six coexisting shrubs. Solid black lines show the fitted linear trend-line, red dashed lines show the null line (temperature offset = 0 °C or below-canopy equals open-field temperature).

The recorded light intensity showed that all the studied shrubs had lower PAR availability at ground level than the grassland (Table S1). Among shrubs, the highest light intensity was recorded under the canopy of *Euphorbia*, especially in summer (Table 1). The other shrub canopies had significantly lower light intensities than the grassland and *Euphorbia* in both seasons. The canopies of *Myrtus* and *Olea* had the lowest light intensities, with also *Juniperus*, *Pistacia* and *Rosmarinus* that cause a significant light attenuation compared to *Euphorbia* and grassland.

Table 1. Light data recorded under canopy of each tree in the shrubland. Data are expressed as percentages and compared to the grassland corresponding to 100%.

	Euphorbia	*Pistacia*	*Olea*	*Juniperus*	*Myrtus*	*Rosmarinus*
Summer	35.03a	3.60b	1.51b	4.61b	1.01b	5.21b
Winter	17.91a	3.71b	4.91b	1.45b	1.02b	1.68b

5.4.3 Chemical parameters correlation with recruitment density

Recruitment density of *Euphorbia* showed non-significant correlations with all parameters, except for the soil sodium Na content, which had a significant positive correlation (Table 2).

Table 2. Pearson's correlation between chemical parameters and recruitment density. Significant differences at $p<0.05$.

Parameters	Density	p-value
pH	-0.18	0.69
Total limestone	-0.53	0.22
Electrical conductivity	0.41	0.36
Chlorides Cl	0.65	0.12
Sodium Na_2O	**0.78**	**0.03***
Organic Carbon	0.47	0.29
Total Nitrogen	0.21	0.65
P	-0.21	0.65
K	-0.37	0.41
Mg	0.03	0.95
Ca	0.07	0.87
Cu	0.36	0.42
Zn	0.34	0.46
Mn	0.54	0.21
Fe	0.20	0.66

5.4.4 Fungal functional guilds correlation with recruitment density

Correlation analysis showed that the relative abundance and richness of all guilds had a weak, non-significant correlation with the recruitment density of *Euphorbia*, including fungal pathogens (r =0.06, p> 0.05), fungal endophytes (r =-0.04, p> 0.05) and fungal saprophytes (r= -0.09, p> 0.05), with the exception of the mycorrhizal fungi (Fig. 5). Specifically, both ectomycorrhizal and arbuscular mycorrhizal fungi showed a significant positive correlation with the recruitment density of *Euphorbia* (r =0.15, p< 0.05; r = 0.14, p< 0.05, respectively).

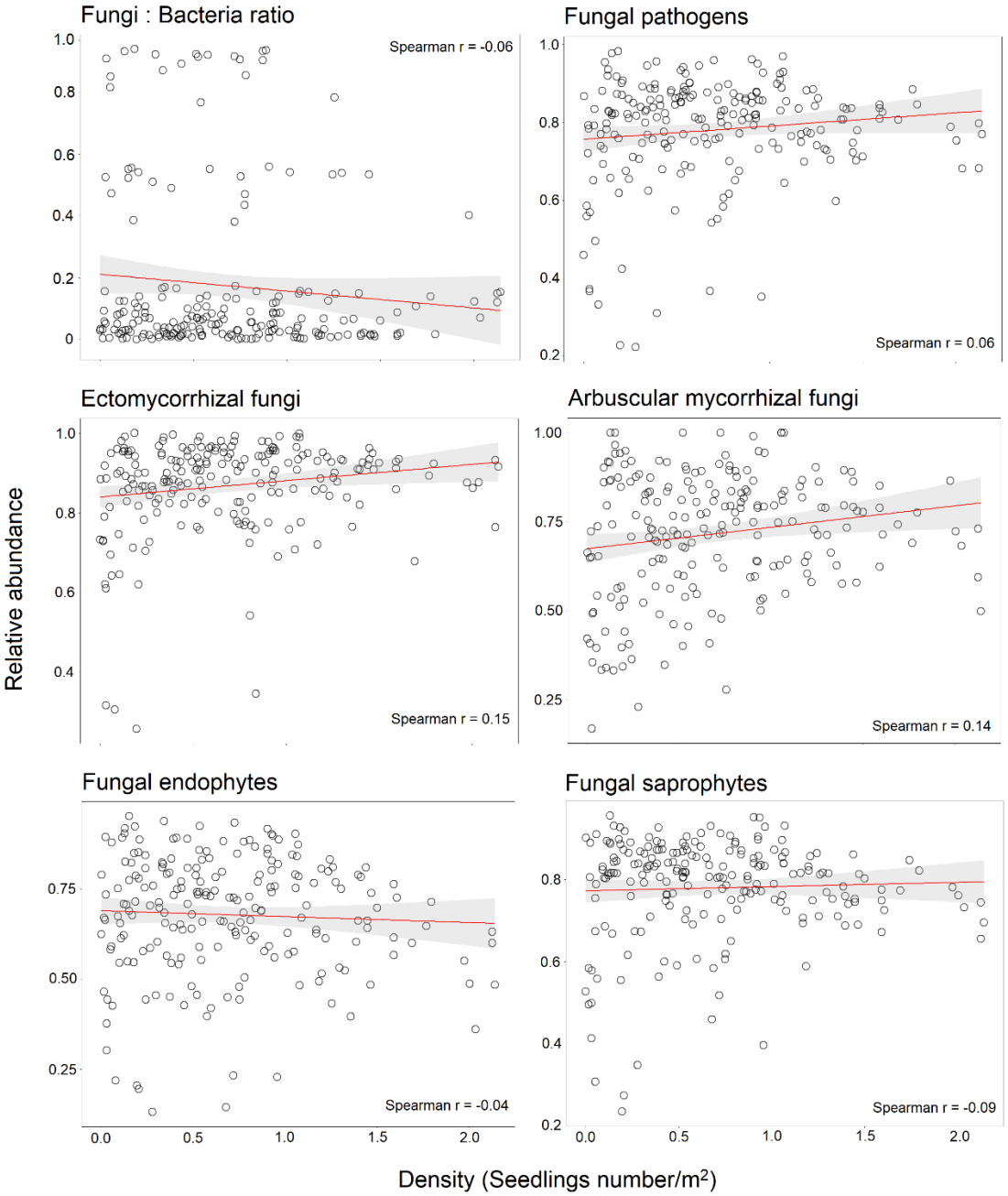

Fig. 5. Relationships between functional fungal guilds and *Euphorbia* recruitment density using a Spearman correlation analysis. Data points show the relative abundance of bacteria and fungi ratio and

fungal guilds in each plot. The relative abundances on the y-axes were scaled from 0 to 1 for better visualization for the fungal guilds.

5.4.5 Correlation network between recruitment density and single ASVs

We constructed two correlation networks between recruitment density and individual bacterial and fungal ASVs after selecting only strong and significant (Spearman's r> 0.6, p> 0.05) correlations based on statistical analysis (Fig. 6). Nodes with high degrees, high Closeness centrality and low Betweenness centrality were considered as keystone taxa. The networks showed that recruitment density was negatively correlated with the following bacterial ASVs: *Gordonia*, *UBA12409*, *Roseococcus* and *OLB12*, while positively correlated with the following ASVs: *Frankiales*, *Truepera* and *Kallotenue*.

Concerning the fungal network, recruitment density was negatively correlated with the following ASVs: *Nectriopsis*, *Sympoventuriaceae*, *Trichosporonaceae* and *Venturiaceae*, while it was positively correlated with the following ASVs: *Iodophanus*, *Trimmatostroma*, *Cadophora*, *Waitea* and *Pyrenochaeta*.

5.5 Discussion

Our results show that *Euphorbia* recruitment is rare under the canopy of conspecifics, but is more common at the edge of the canopy and increases thereafter, which is consistent with the JC model. Remarkably, all of the few seedlings found under *Euphorbia* conspecific canopy were less than 2 years old, while those found outside the canopy reached an age of more than 10 years. The distribution of recruitment with a peak at an intermediate distance from the root point of adult conspecifics and the outward shift in recruitment age from the seed source, a pattern previously reported in forests (Augspurger, 1983) and shrublands (Bonanomi et al. 2008), are all indicators of strong self-inhibition overwhelming the seed dispersal kernel. In addition, we found that *Euphorbia* recruitment density was much higher under some heterospecific tree canopies, i.e. *Olea* and *Juniperus*, intermediate in grasslands and under *Myrtus* and *Pistacia*, and very low under *Rosmarinus* and conspecifics, highlighting the species-specificity of recruitment requirements.

We then investigated the causes of the reduced success of conspecifics near adult individuals that cause the formation of the "exclusion zone". First, we investigated the role of light availability on the ground, which is often important for seedling recruitment and

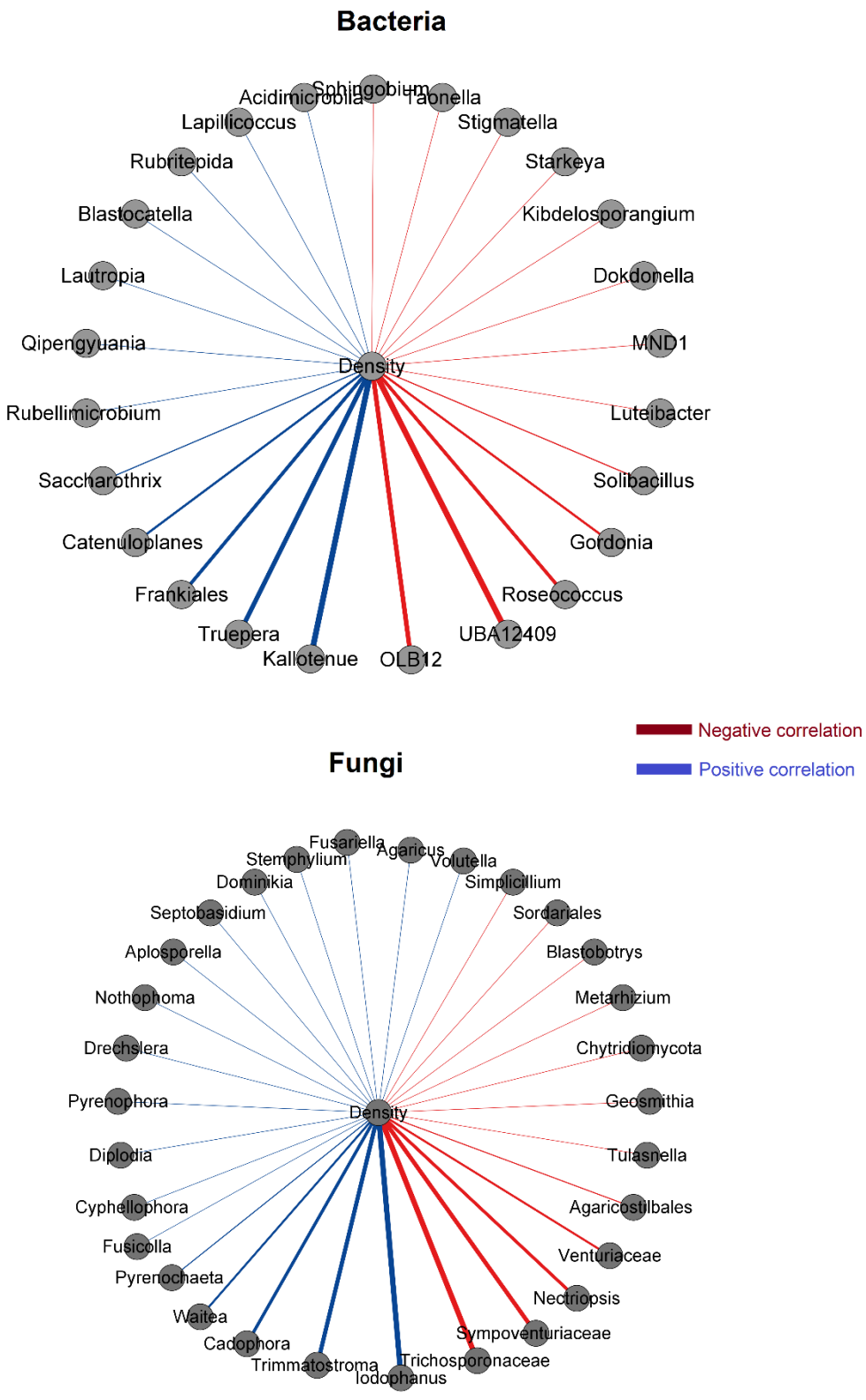

Fig. 6. Correlation base network between bacterial (above) and fungal (below) ASVs and *Euphorbia* recruitment density. Edges connecting nodes show either positive (light blue) or negative (red) connection relationships. Edges width represents correlation strength, the connection stands for significant correlations ($p<0.05$).

establishment in Mediterranean climates (Urbieta et al. 2008). Our results showed that light intensity was significantly higher under the canopies of *Euphorbia* than under the canopies of the evergreen heterospecifics, but largely lower than in the adjacent open grassland. Remarkably, recruitment of *Euphorbia* occurred at higher density under open grassland, where light intensity was highest, than under the canopies of conspecifics, but also under some evergreen shrubs such as *Olea* and *Juniperus*, which cast very deep shade throughout the year. Thus, our data suggest that *Euphorbia* can successfully recruit over a wide range of light availability, ruling out the hypothesis that the exclusion zone under adult conspecifics is caused by either low or excessive solar radiation.

In addition to light resources, recruitment establishment is also influenced by the microclimate under the plant canopy. In Mediterranean climates, high temperatures and direct solar radiation can hamper seedling establishment, especially when accompanied by prolonged dry periods (Breshears et al. 1998; Callaway, 2007; Stinca et al. 2015). We found that all shrubs buffered high temperatures in summer, as well as on sunny autumn days, as indicated by the offset within canopies compared to the grassland. In agreement with the results of De Frenne et al. (2019), the cooling effect of the understory is higher when ambient temperatures are hot, as is the case in summer and on sunny days. Moreover, we found not only that understory versus grassland temperature offset is stronger when temperatures become higher, but also that the buffering effect is more evident for evergreen shrubs than for *Euphorbia*. Shrub canopies could thus reduce the severity of high temperatures impacts on recruitment establishment. However, recruitment of *Euphorbia* was higher, compared to under conspecific canopy, in open shrubland as well as under shrubs with stronger temperature buffering, i.e. *Juniperus* and *Olea*. As shown for light availability, *Euphorbia* also appears to have the ability to recruit in relation to air temperature in both buffered environments and open ground areas. Indeed, our data do not support the hypothesis that variations in microclimate explain the exclusion zone observed among adult conspecifics.

Habitat heterogeneity and species-specific habitat preferences can lead to spatial clustering and localised distributions and should therefore be considered when trying to understand the local distribution of species and the relative importance of distance dependence (Piao et al. 2013). In our study case, soil chemical parameters did not appear to be responsible, although there were large differences in soil fertility among different shrubs (Idbella et al. 2022). In fact, we found that *Euphorbia* recruitment density was not related to soil organic matter and major nutrients such as N, P and major cations. Previous studies in Mediterranean climates reported the presence of an exclusion zone under conspecific canopy of nitrogen-

fixing shrubs such as *Medicago marina* (Bonanomi et al. 2008) and *Genista aetnensis* (Stinca et al. 2015) despite the noticeable island of fertility that occurs under the canopy. Our results showed a positive correlation between soil Na concentration and recruitment density of *Euphorbia* as the only exception. Similar to our results, Pule et al. (2018) showed that soil sodium content was positively correlated with *Seriphium plumosum* recruitment density. While some studies consider Na toxic to plants and even insects and animals at high concentrations (Findlay & Kelly, 2011), other studies have shown that this micronutrient plays a positive role in the decomposition of terrestrial plants by catalysing the utilisation of N and P by soil invertebrates (Kaspari et al. 2017). Recently, Bonanomi et al. (2021) reported that Na is a limiting factor for wood debris decomposition in Mediterranean climates. Indeed, Na is essential for plant growth, but usually in very small amounts, and is not considered as a fertiliser in agroecosystems. Therefore, the positive correlation found could probably be spurious, but further studies are needed to clarify the role of Na availability in shrub recruitment.

Excluding light availability, microclimate and soil fertility, our last hypothesis to explain the observed JC distribution is related to the soil microbiota and, in particular, the accumulation of specific pathogens under adult conspecifics (Packer & Clay, 2000). Overall, we found no negative correlation between *Euphorbia* recruitment and soil pathogens here, except for the genus *Nectriopsis*. In detail, we found no correlation with fungal pathogens as a functional group, but also considering individual ASVs such as *Alternaria*, *Cladosporium*, *Stemphylium* and *Fusarium*. Our results show that at the low taxonomic level, we recorded high abundance of the genus *Claviceps* in grassland soil, while it was almost absent under all shrub species. This result suggests a rather specific link between *Ampelodesmos* and *Claviceps*, which are known for ergot disease infecting ~200 species of wild and cultivated grasses (Boestfleisch et al., 2015). In addition, *Euphorbia* was characterised by the presence of a considerable amount of weak saprophytes, including *Aspergillus*, *Aureobasidium*, *Alternaria*, and *Cladosporium*, while *Rosmarinus* exclusively harboured the genus *Didymella*, which are opportunistic parasitic microorganisms that often exploit special conditions to colonise plants and occasionally cause severe damage (Blancard, 2012). Furthermore, the soil of *Pistacia* contained large amounts of *Arthrinium*, a globally distributed pathogenic genus with a broad host range (He & Zhang, 2012); whereas *Juniperus* exclusively harboured *Blumeria*, obligate biotrophic pathogens that cause destructive foliar diseases of many plant species (Feng et al. 2009). Several plant pathogens are indeed not specific to *Euphorbia*, but in our detailed analysis, the only significant strong negative correlation with exclusion zone under conspecifics was found with *Nectriopsis*, a relatively common genus found on a range of

hardwood trees and woody shrubs in temperate regions. It is sometimes considered a plant pathogen that causes various diseases depending on the species, such as "coral spot" caused by *N. cinnabarina* (Hirooka et al. 2011) and canker disease caused by *N. galligena* (McCracken et al. 2003). However, this genus was also found to occur among *Pistacia* and *Rosmarinus* soils, refuting the theory that it could be the cause of the observed pattern under *Euphorbia* conspecifics.

Ectomycorrhizal fungi (EM) can reverse JC distribution by supporting recruitment under and near conspecifics (Dickie et al. 2005). Evidence suggests that indirect support for this emerge by monospecific stands in temperate and tropical forests are generated by EM associated tree species (McGuire, 2007; Ebenye et al. 2017). In other words, EM associations can counteract negative distance-dependence through positive plant-soil feedbacks that arise from protection against pathogens and provision of additional nutrient resources (Segnitz et al. 2020). Consistent with this, recent experimental studies suggest that AM trees experience stronger antagonism from their associated soil microbiota compared to EM shrubs and trees (Bennett et al. 2017; Kadowaki et al. 2018; Teste et al. 2020). Our results showed a slight significant positive correlation between both EM and AM with recruitment density. In our study, no shrubs form a symbiosis with EM, except *Juniperus* (Mejstrik & Cudlin, 1983), so a direct positive effect of mychorrizal network seems unlikely. Instead, the weak but positive correlation between AM fungi and recruitment density suggests that fungi may partially explain the abundance under some shrubs, but not the inhibition under conspecifics. In this context, Teste & Laliberté (2021) found higher seedling survival under conspecific trees compared to heterospecific trees in a Mediterranean shrubland in Australia, contradicting the Janzen-Connell distribution pattern.

A more detailed co-occurrence analysis identified some positive and negative associations between specific ASVs and recruitment density. Specifically, we found that *Euphorbia* adults generate a specific microbiome fingerprint under their canopy, with *Rubrobacter, Bacillus, Mycobacterium, Streptomyces,* and *Pirellula* as abundant bacteria and *Penicillium, Aureobasidium, Alternaria*, and *Fusarium* as abundant fungi. For bacterial ASVs, we found strong negative correlations between density and a number of ASVs, including OLB12, UBA12409, *Rosecoccus,* and *Gordonia* species. None of these bacterial ASVs are known in the literature, with the exception of *Gordonia*, an opportunistic human pathogen (Sowani et al. 2017). In contrast, strong positive correlations have been found between recruitment density and many bacterial ASVs such as *Truepera*, *Kallotenue* and *Frankiales*. None of these ASVs are known to have a specific function in soil, with the exception of the

order *Frankiales*, which includes nitrogen-fixing organisms and could produce cellulose-degrading enzymes (Guan et al. 2014). Thus, our results suggest that some bacteria may contribute to distance dependence inhibition of *Euphorbia* seedlings, but more data on the functional ecology of the poorly studied species are needed.

In fungi, strong negative correlations were found between several ASVs and recruitment density. In particular, *Trichosporonaceae*, a family of *Basidiomycota* yeasts found in soil (Yurkov, 2018), in addition to some oligotrophic fungal ASVs such as *Sympoventuriaceae* and *Venturiaceae*, which are mainly endophytes (Jumpponen & Jones, 2009). Similarly, several fungal ASVs showed strong positive correlations with seedling density, such as *Iodophanus*, *Trimmatostroma*, *Cadophora* and *Verrucocladosporium*. All observed correlated ASVs had no function in the literature, with the exception of *Cadophora*, a widespread soil fungus previously associated with several plants (Tedersoo et al. 2009). Their ecological roles range from plant pathogens to mutualistic partners (Smith & Read, 2007). The observed positive correlation between these genera and recruitment density confirms their role as mutualistic partners. Thus, our results suggest that the observed Janzen-Connell pattern of *Euphorbia* species cannot be explained by the accumulation of species-specific pathogens in the soil because, considering the low taxonomic level, there was a clear negative strong correlation between seedling density and the pathogenic *Nectriopsis*, but this pathogen was also abundant under other shrubs, i.e. *Pistacia* and *Rosmarinus*, which showed a much better recruitment pattern than under the conspecifics.

5.6 Conclusion

Overall, our field study provides evidence for a community-wide Janzen-Connell effect of *Euphorbia dendroides* in this Mediterranean shrubland, as we found greater recruitments under heterospecifics compared to under conspecifics. However, in contrast to the mechanism proposed by JC, our results showed that the observed pattern could not be explained by the accumulation of species-specific pathogens in the soil, nor by changes in soil nutrients, organic carbon content and microbial properties. Moreover, the microclimate under the shrub canopies could not explain the observed JC pattern either, as all canopies showed a buffering effect on temperature, with a cooling effect in summer and on hot days and a limited warming effect in winter. Our study highlights the need to consider a more complex and context-dependent mechanism involving autotoxicity theory to improve our understanding of mechanisms of conspecific distance dependence and species coexistence. Autotoxicity theory states that when

conspecific litter decomposes, some allelochemicals, including self-DNA, are released into the soil and cause specific self-inhibition for young seedlings.

6 Chapter 6: Soil microbial and chemical legacies drive the plant-soil feedback of eight major crops belonging to two functional groups, grasses and legumes

6.1 Abstract

Plants can affect the soil in which they grow, and via these changes they can positively or negatively affect other plants that later grow in that soil, a phenomenon called plant-soil feedback (PSF). PSFs are generally shaped by chemical and microbial legacies of plants in the soil. The importance of PSFs in understanding ecosystem functioning is the focus of much recent research, particularly in predicting consequences for agricultural production. Thus, one intriguing possibility is to use positive PSF effects in sustainable agriculture to promote plant growth and pathogen resistance. For this reason, we grew eight different plant species belonging to two functional groups, including four grasses and four legumes, to condition living soil. After the conditioning phase, the same species were sown as response plants in a combination that allowed each plant species to grow on the conditioned conspecifics and heterospecific soils. To determine the effect of conditioning on biotic and abiotic factors in the soil, we used high-throughput sequencing in conjunction with soil chemical analyses. The results of the overall feedback effect showed that *Glycine* had the strongest negative feedback, followed by *Triticum*, while low feedback was found for *Lolium*. On the other hand, *Lens* was the only crop that showed strong positive feedback, while slight positive feedback was observed for both *Zea* and *Medicago*. As for soil chemistry, *Glycine* conditioned soil had the highest content of OM, total N, Mg and Fe, while the content of Mn, Cu, Ca, K, P, Na, Cl and soil pH were significantly low. In addition, our results showed that microbial diversity had no significant differences among the eight conditioned soils. The effect of conditioning on microbial community composition showed no specificity between the two plant functional groups. However, the abundance of functionally important microbial phyla was affected by each plant species. In addition, our results suggest that each plant species conditioned its own soil with a high proportion of putative pathogenic fungi. However, when linking the conspecific biomass to different taxa present in the soil, our co-occurrence analysis showed that all plant species had a strong significant negative correlation with fungal pathogens accumulated in the soil, except for *Glycine*, which had a strong negative correlation with plant growth-promoting rhizobacteria such as *Arthrobacter* and *Bacillus*. This suggests that the ability to predict PSFs requires a better understanding of plant interactions with diverse communities of plant pathogens and mutualists, rather than with individual host-specific pathogens.

Keywords: plant-soil feedback, functional groups, high-throughput sequencing, soil chemistry, pathogens, rhizobia

6.2 Introduction

Plants can differentially influence their soil environment by altering its physical, chemical, and biological features, thus affecting their performance relative to their competitors, and ultimately leading to changes in plant community composition and diversity (Bever et al. 1997) through a belowground process called plant-soil feedback (PSF). A particular plant species may alter its soil environment in a way that increases its own growth rate compared to other plant species, resulting in a positive feedback, or in a way that decreases its own growth rate relative to that of other plant species, resulting in a negative feedback (Van der Putten et al. 2013). Positive PSF may result from improved nutrient availability (Grayston et al. 1998; Chapman et al. 2005) or the accumulation of symbiotic mutualists in the rhizosphere (Klironomos, 2002). Negative PSF may be due to the immobilization or depletion of nutrients (Berendse, 1994), to the build-up population of root herbivores and soil pathogens (Van der Putten, 2003) or accumulation of autotoxic factors (Cesarano et al. 2017). PSF describes the net effect of these concurrent events, i.e. positive and negative effects, as they do not occur in isolation (Harrison & Bardgett, 2010). Variations in the strength of PSFs between species can predict the distribution of species abundance; with rarer species generally have more negative PSF (Klironomos, 2002; Bennett et al. 2017).

Among the many mechanisms that cause PSF, the two most noted are plant-mediated nutrient cycling (e.g. abiotic factors) and plant-microbial interactions (e.g. biotic factor). Plants excurse different effects on local nutrient cycling, and studies often suggest that litter decomposability is an important plant trait controlling plant-mediated nutrient cycling (Berendse, 1994; Miki & Kondoh, 2002). In natural ecosystems, litter may leave physical, chemical and biotic legacies in the soil that have a strong impact on soil functioning and plant growth (Ehrenfeld et al. 2005; Elgersma et al. 2012). In particular, the production of nutrient rich, lignin poor litter that decomposes rapidly creates positive PSF, by promoting nutrient cycling, especially when the benefits act more strongly on the plant itself (Lehmann & Kleber, 2015). On the other hand, direct interactions between plants and soil microbes show that plants differ in their local microbial communities and their response to specific microbial species (Bever et al. 2010; van der Putten et al. 2013). The main categories of soil microbiota that characterise PSF are enemies (i.e., soil microbial pathogens), symbionts (i.e., mycorrhizal fungi, endophytes, nitrogen fixing and plant growth-promoting microbes), and decomposers (i.e., microbiota that degrade litter, root exudates, and soil organic matter) (Wardle, 2002). They can all influence plant growth directly and indirectly through their influence on soil

physicochemical properties (Ehrenfeld et al. 2005). A positive PSF can occur when the plant promotes population growth of symbionts in different ways compared to enemies during cultivation (Klironomos, 2002) or when the promoted enemies have stronger effects on competitors than on the plant itself (Bever et al. 2010). Negative PSF occurs when the plant differentially suppresses population growth of symbionts compared to enemies, or when facilitated symbionts have stronger effects on competitors than on the plant itself. Abiotic PSF effects are likely to be less species-specific (Aerts & Chapin, 2000), whereas biotic PSF effects may be highly specific (Van der Putten, 2003). Another important factor cited in the literature to explain the increase in negative PSF is the release of autotoxic compounds during decomposition of plant litter (Bonanomi et al. 2005). By definition, autotoxicity causes negative PSF, by inhibiting the growth of conspecifics. In some cases, autotoxic chemicals also inhibit mutualistic microbes and neutralize positive PSF (Zhou et al. 2018). However, two main criticisms of the autotoxicity hypothesis have been raised. The first states that toxins from plant residues are rapidly degraded by microbial activity in the soil and become ineffective after a few weeks, while negative PSF may remain in the field for months or even years; the second states that many, if not all, organic compounds extracted from diseased soils and plant residues exhibit general phytotoxicity, which is in contrast to the species-specificity of negative PSF. Alternatively, and in a more recent study, Mazzoleni et al. (2015) reported that fragmented extracellular self-DNA accumulated in litter during decomposition of conspecific residues has species-specific inhibitory effects on various wild plants. These results provide a chemical basis for autotoxicity that must be considered in explaining the negative PSF.

In agroecosystems, ecological resilience and resistance can be enhanced by improving system diversity through crop rotation, intercropping, cover crops or integration of cropping and livestock (Liebman & Schulte, 2015; Murrell, 2017). It has long been known that PSFs influence agricultural production and form the basis for crop rotation (van der Putten et al. 2013). Negative PSF due to the accumulation of plant pathogens often leads to yield decline in continuous monoculture farming. Crop diversification, including intercrops and rotations, reduces the incidence of soil pathogens by breaking their cycle (Letourneau et al. 2011) and improves soil microbial biomass and functions, including beneficial microbiomes such as arbuscular mycorrhizal fungi (AMF) (Lacombe et al. 2009) and nitrogen fixers (Li et al. 2016). Moreover, crop rotation induces changes in nutrient cycling processes (McDaniel et al. 2014), which can also result in variable indirect effects on pathogens and mycorrhizal fungi. For instance, increased nutrient availability might stimulate pathogen growth due to increased host

plant productivity and tissue quality (Nordin et al. 2006). However, the effects might suppress mycorrhizal fungi due to shifts in the nutrient limitation status of the microbes (Treseder & Allen, 2002; Johnson, 2010). Mechanisms for this influence involve variation in litter chemistry, soil pH and nutrient contents among crops (Fierer & Jackson, 2006). Recently, research has shown that the direction, i.e. positive or negative, and magnitude of PSFs are influenced by the agricultural management system (Johnson et al. 2017) and the phylogenetic distance between interacting species (Miller & Menalled, 2015). For example, legume-grass mixtures, an essential element of crop rotation, especially for organically managed farms in the temperate climate of Europe (Grüner et al. 2020). Grass monocultures have often been preferred by producers because weeds and grazing can be easily controlled (Beuselinck et al. 1994). However, the cost of nitrogen fertilizer and the potential negative environmental impacts of nitrogen application have led to an urgent need to maintain or increase pasture production while reducing nitrogen fertilizer use (Solomon et al. 2011). This has led to increased interest in grass-legume mixed pastures. Legumes, as nitrogen fixers, can increase nutrient availability to other plants, producing positive PSF effects (Harrison and Bardgett, 2010). Similarly, grasses with highly branched roots may provide a more suitable habitat for root-associated microbes that have positive effects on other plants (Latz et al. 2015). Of course, an increase in root surface area, which is common in grasses, could also lead to an increase in the abundance of plant antagonists such as root pathogens, but root pathogens of grasses are specialized on monocots and are unlikely to affect plants from any other functional group negatively (Cortois et al. 2016). However, the presence of grass endophyte symbioses can affect legume establishment (Stevens & Hickey, 1990). For example, negative effects have been reported in *Medicago sativa* L., *Trifolium pratense* L., *Lotus tenuis* L., and *Trifolium repens* L. when grown with endophyte-infected tall fescue (Hoveland et al. 1999; Liu et al. 2021). These studies suggest that these negative effects are caused by the presence of endophytes and their influence on the competitiveness of the host grass, as they can alter several host traits that may affect legume success and their interaction with rhizobia and AMF (García-Parisi et al. 2017; Idbella et al. 2019, 2021). Therefore, it is important to investigate the mechanisms by which grasses can inhibit legume establishment.

Most studies that have investigated feedback effects within agroecosystems have examined the performance of crops for one or two species in soils conditioned by conspecifics and heterospecifics (Van der Putten et al. 1993; Bever, 1994; Klironomos, 2002; Seipel et al. 2019). As a result, little is known about how soil conditioning affects plant performance in

agroecosystems (Ehrenfeld et al. 2005; Bezemer et al. 2006). In the present study, we investigated how individual plant species promoted or inhibited conspecific and heterospecific growth via changes in the soil. Specifically, we grew eight plant species, including four grasses, e.g., *Triticum durum* L., *Zea mays* L., *Lolium perenne* L., *Oryza sativa* L., and four legumes, e.g., *Medicago sativa* L., *Glycine max* L., *Lens culinaris* L., and *Trifolium repens* L. The pots were exposed for the duration of conditioning over one year. After the conditioning phase, all plant communities were removed from the soil and the same species were sown as response plants in a combination that allowed the growth of each plant species on the conditioned conspecifics and heterospecific soils. While most studies testing the feedback effect use the method of soil sterilization (i.e., comparing growth of response plants on non-sterilized vs. sterilized conditioned soil) or the method of adding soil inoculum to sterilized background soil to assess the role of soil microbiota (i.e., comparing growth of response plants on conditioned soil with vs. without soil organisms), here we have used a "whole feedback" approach that is based on conditioning the soil and grew the response phase in the untouched soil. This method resemble real field conditions where during condition not only microbiota is changed but also soil chemistry. Therefore, with this approach, both changes in soil biota and chemistry contribute to PSF. Soil chemical properties as well as soil fungal and bacterial community were characterized by next-generation sequencing. The aim of this study was therefore to test the effects of the different soil legacies established during the conditioning phase by each plant species on the chemical and microbial properties of the soil and, consequently, on the growth of conspecifics and heterospecifics during the response phase. Our hypothesis was each species suffer species-specific negative PSF while legumes cause positive PSF on grasses and the reverse. Specific aims were:

i. to provide evidence that in the response phase, grasses and legumes would grow less in soils that were dominated by their own functional type;
ii. to assess if the nature of negative PSF is associated with soil pathogens accumulation;
iii. to assess if positive PSF is caused by increased soil N and other major nutrients.

6.3 Material & Methods

The experiment was divided into two phases: the conditioning and the response phase (Fig. 1). In the conditioning phase, eight crop species were used to condition soil individually: *Triticum*

durum L., *Zea mays* L., *Lolium perenne* L., *Oryza sativa* L., *Medicago sativa* L., *Glycine max* L., *Lens culinaris* L., and *Trifolium repens* L. In the response phase, six crop species were grown in a soil previously conditioned by the same crop (i.e., conspecific) or by each of the other seven crop species (i.e., heterospecifics) for a full growth cycle. The seeds used in this experiment were collected after growing the commercially purchased seeds with no previous treatment (De Corato sementi^{R}).

6.3.1 Conditioning phase

In this phase, the plants are grown in the soil for a certain period to condition it and to change the local biotic and abiotic soil conditions (Ehrenfeld et al. 2005; Van der Putten et al. 2013). Pots (20 cm opening diameter ∗ 18 cm height ∗ 15 cm bottom diameter) were filled with sterile soil with the following properties: 22.1% clay, 56.6% silt, 21.3% sand, pH 7.74, electrical conductivity 0.32 dS m^{-1}, organic carbon 15.4 g kg^{-1}, total nitrogen 1.6 g kg^{-1}, C/N ratio 9.6, $CaCO_3$ 7.16 g kg^{-1}, available phosphorus 239 mg kg^{-1}. Soil was collected from the topsoil (0-30 cm) of a farm in the Campania region, southern Italy (E: 14° 18', N: 40° 51'; 4 m a.s.l.). It was homogenized and sieved (1 cm mesh size) to remove coarse fragments and all macro-arthropods. The soil was sterilized before the start of the experiment by autoclaving at one atm pressure and 120 °C for 1 h, three times at 24 h intervals.

In total, the conditioning phase included 240 pots (monocultures of 8 plant species × 30 replicates for each plant species). The replicate pots of each species in the conditioning phase were kept separate as eight individual blocks throughout the experiment. Fifteen seeds were sown in each of the conditioning pots for each plant species. Seeds were surface sterilized in a 3% sodium hypochlorite solution for 1 min and rinsed several times with sterile water before use. After germination, the number of seedlings in each pot was reduced to five and the pots were watered regularly. After a year of soil conditioning, including the time when we stopped watering and allowed the plants to dry out in their pots, the plants were carefully removed from each pot and the roots were left in the soil because the rhizosphere around these roots may contain a large portion of the microbial rhizosphere community.

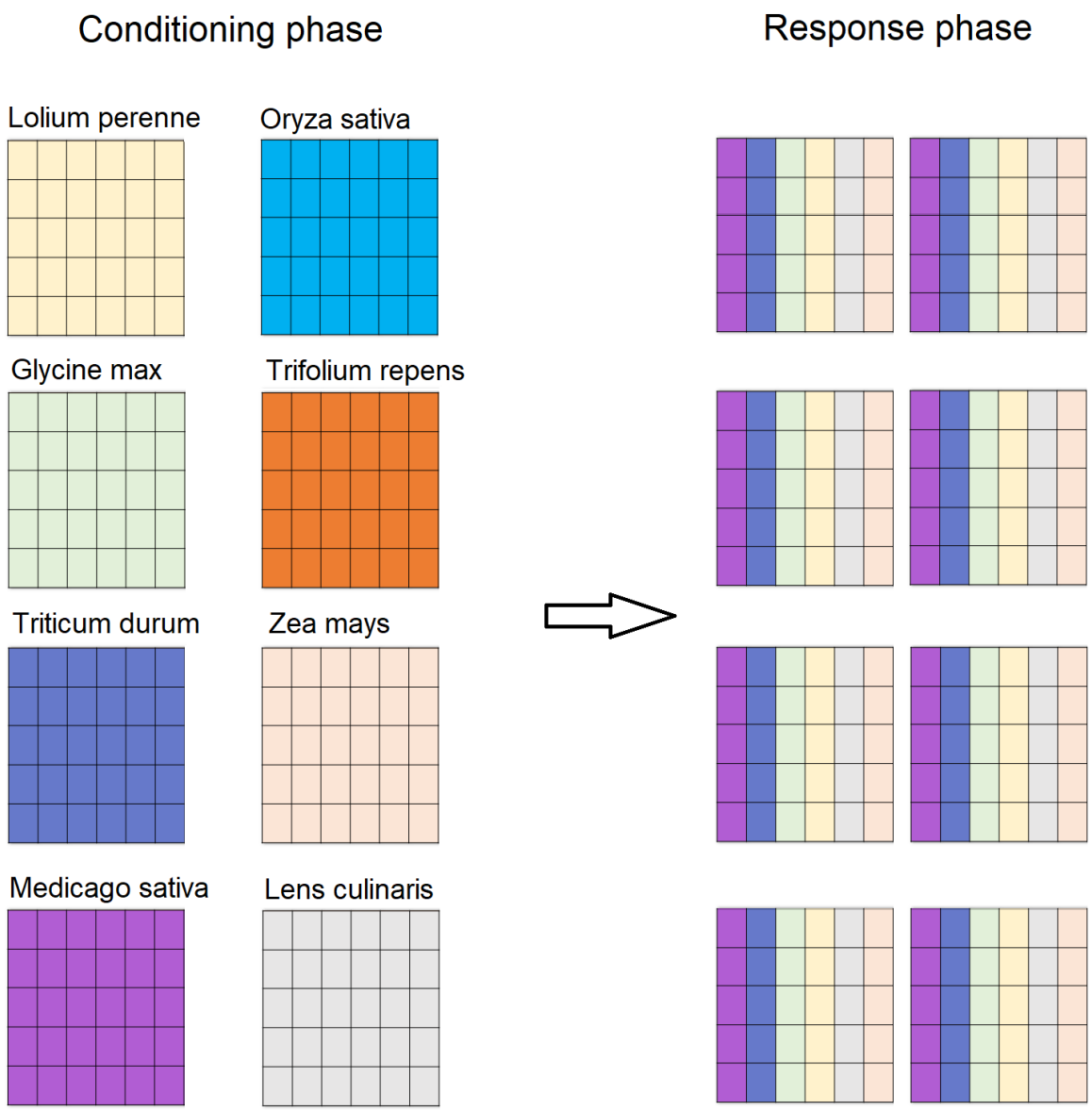

Fig 1. Conceptual representation of the experimental design. Soil was conditioned by monocultures of eight crops. The eight soil conditioning treatments are identified by having different colours of the soil squares. Thirty independent soil replicates were made for each of the conditioned soils. After the conditioning phase, five replicates of each of the conditioned soils were used to grow each of the six response crops, resulting thus in a combination of six response crops growing in each of the eight conditioned soils. For more details, see the main text.

6.3.2 Response phase

In this second phase, each conditioned soil by plant species composed of a block of 30 pots was divided into 6 sub-blocks of 6 replicates (Fig. 1). Fifteen seeds were sown in each response pot using the same seed treatments as in the conditioning phase. Accordingly, in this phase we obtain response plants growing in soils previously conditioned by the same plants as well as by seven heterospecifics. 240 conditioned pots were used (6-response plants × 5 replicates × 8

conditioned blocks). After germination, the number of seedlings in each pot was reduced to five and the pots were watered regularly every 5 days. Seven months later, all plants were harvested. Plants were cut at soil level, shoots were dried at 70 °C for 72 h, and their dry weight was recorded. For each plant block, the soil from the four unused pots was collected and then divided into two fractions: one fraction was stored at 4°C to study the chemical properties of the soil; the other fraction was stored at -20°C and used for molecular analysis.

6.3.3 Soil chemistry

After the conditioning phase, soil samples were dried in a ventilated chamber at room temperature until a constant weight was reached. The soil was analysed for 15 parameters, namely total organic carbon, pH, total nitrogen and macro and micronutrients important for plant growth. Specifically, the following parameters were measured: soil electrical conductivity (EC) and pH were determined in soil-water suspensions at a ratio of 1:5 and 1:2.5, respectively, using a conductivity meter and a pH meter (Czekała et al. 2016). Total nitrogen was determined using the Kjeldhal method (Czekała et al. 2016), while phosphorus was determined using the molybdovanadate-phosphate method (AOAC, 1990). Organic matter (OC) content was determined by weight loss at 550°C for 8 h (Silva et al. 2014). Potassium, magnesium, iron, manganese, calcium, sodium, copper and zinc were determined by flame atomic absorption spectroscopy (Peters et al. 2003). Total limestone is determined by the weight method against a strong acid. Attack of the limestone results in gas release of CO_2, the volume of which is measured (LANO: NF ISO 10693). Finally, the chloride content (Cl) in the soil was determined by the volumetric method described by Meldrum and Forbes (1928).

6.3.4 Microbial DNA extraction and amplicon sequencing

The microbiome of three soil replicates of each plant species after the conditioning phase was analysed by Illumina high-throughput sequencing. The DNeasy PowerSoil kit (Qiagen) was used to extract the microbial DNA from 5 g of each homogenized soil. Bacterial and fungal diversity were assessed by high-throughput sequencing of the amplified V3-V4 regions of the 16S rRNA gene (~460 bp) and ITS1-2 (~300 bp). PCR was carried out with primers S-D-Bact-0341-b- S-17/S-D-Bact0785-a-A-21 (Berni Canani et al. 2017) and BITS1fw/ B58S3- ITS2rev (Bokulich & Mills, 2013) using conditions reported in the original studies. For bacterial primers S-D-Bact-0341-b-S-17 (5' -CCTACG GGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5' -GAC TACHVGGGTATCTAATCC-3'), PCR conditions were: 25 cycles of 95°C for 3 min, 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, 72°C for 5 min and held at 4°C. For fungal primers BITS1fw (5'-ACCTGCGGARGGATCA-3') and B58S3-ITS2rev (5'-

GAGATCCRTTGYTRAAAGTT-3'), PCR conditions were: 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and a final extension of 72°C for 5 min. PCR products were purified with the Agencourt AMPure beads (Beckman Coulter, Milan, IT) and quantified using an AF2200 Plate Reader (Eppendorf, Milan, IT). Equimolar pools were obtained and sequencing was carried out on an Illumina MiSeq platform, yielding to 2× 250 bp, paired-end reads.

Bacterial and fungal sequences were processed using the DADA2 package (version 1.16.0 pipeline) (Callahan et al. 2016) in R software (4.0.4) (Team, 2016). DADA2 provides better taxonomic resolution than other methods by retaining unique sequences and calculating sequencing error rates rather than clustering to 97% similarity (Hugerth & Andersson, 2017). The resulting taxonomic units are referred to as amplicon sequence variants (ASVs) rather than operational taxonomic units (OTUs). For bacterial sequences, forward and reverse reads were trimmed to 240 bp, and primer sequences were removed from all reads. The following filter parameters were used: maxN = 0, maxEE for both reads = 2, truncQ = 2 (MaxEE corresponds to the maximum expected errors, TruncQ represents the parameter that truncates reads on the first occurrence of a quality score of less than or equal to two, and MaxN is the maximum number of 'N' bases accepted). Error rates were estimated with learnErrors using nearly 4 million reads. Sequences were dereplicated using derepFastq with default parameters and exact sequence variants were resolved using the dada algorithm. The RemoveBimeraDenovo function was then used to remove chimeric sequences. For fungal sequences, the pipeline was pre-empted by a preliminary step of trimming adapter sequences and low quality ends (<Q20) using Cutadapt software (Martin, 2011). For both the bacterial and fungal datasets, reads with more than three errors in the forward reads and five errors in the reverse reads were removed. Taxonomy was then assigned using assignTaxonomy based on the SILVA (v132) and UNITE (v7) databases for bacterial and fungal communities, respectively (Quast et al., 2013; Nilsson et al., 2019). *Chloroplast* and *Streptophyta* contaminants and singleton ASVs were removed, and relative abundances of the other taxa were recalculated.

6.3.5 Statistical analysis and data visualisation

The statistical significance of the biomass data obtained from the experiment was evaluated using two-way analysis ANOVA (analysis of variance) to determine the main and interactive effect of the fixed factors of conditioning and response phase on shoot biomass. The results of the analysis of variance were furtherly validated by the pairwise Tukey test comparing the individual means of response plants in each soil history. Furthermore, the plant-soil feedback effect has been calculated as the ratio between the total biomass of the conditioned and response

soil (Brinkman et al. 2010). In the present experiment, the feedback effect was calculated as ln (total biomass of a response plant growing in soils conditioned by the same plant / total biomass of the same response plant growing in soils conditioned by a different plant). To test for significant changes in the feedback effect, the interaction data were analysed using a generalised linear model, i.e. GLM, which includes conditioning and response status as fixed factors.

For the microbial data, alpha diversity metrics were calculated and heatmaps were constructed using PRIMER 7 software (Primer-E Ltd, Plymouth; UK) to assess variation in community composition at the lowest taxonomic levels. The heatmaps were used to represent the 50 most abundant taxa in the fungal and bacterial communities and to cluster the variables according to the results of an association similarity index. Based on a resemblance matrix calculated using Bray-Curtis dissimilarity, non-metric multidimensional scaling (NMDS) analyses based on the abundance of the microbial communities were performed using the "meta.mds()" function of the vegan package implemented in R (version 4.0.4). The vector fitting of environmental variables to NMDS ordination was determined using the "envfit()" function of the vegan package with 15 major components of physical and chemical characteristics. The significance of changes in composition between the two microbial communities was tested using PERMANOVA (999 permutations), using the conditioning plant species as a fixed factor. The significance of variation in the alpha diversity metrics of the two microbial communities was assessed along with the soil chemical characteristics using the ANOVA test and the means were pairwise separated using the *post hoc* Tukey test to provide further details on the level of significance between the samples. Moreover, we applied a Spearman ranking correlation test to compare the shoot biomass of each of the six response species with soil chemical attributes, and a heatmap was generated using Rstudio (ComplexHeatmap package). The level of significant differences was evaluated with $P < 0.05$. All statistical analyses were performed using STATISTICA 13.3 software.

In addition, co-occurrence networks were established with bacterial and fungal communities based on individual ASVs to assess co-occurrence or potential interactions between species and response plant biomass. To reveal the complexity of the microbiome and potential interactions among microbial community members with the biomass, network analyses were conducted for the communities of the six different conditioned soils sampled. Recent studies have shown that network inference techniques are useful to decipher microbial relationships based on co-occurrence patterns (Barberan et al. 2012). To focus on the most

abundant ASVs and reduce the impact of rare ones, only the 50 most abundant ASVs were analysed for each bacteria and fungi. Pairwise correlations between ASVs and biomass were calculated using Spearman's correlation in R (Hmisc package). Based on statistical analysis, only strong and significant (Spearman's $r > 0.6$ or $r<-0.6$ and $p < 0.05$) correlations were considered. The network visualisation was created using Gephi (version 0.9.2, Bastian et al. 2009). Each edge represents a robust and significant correlation and each node represents an ASV together with the biomass node.

6.4 Results

6.4.1 Crop response to soil conditioning

Shoot biomass showed significant differences depending on the combination of conditioning and response status (Fig. 2). Specifically, shoot biomass of *Lolium* was significantly lowest when the soil was conditioned with *Triticum, Zea* and *Lolium* itself, while it was highest when the soil was conditioned with *Medicago* and *Trifolium*. Shoot biomass of *Lens* was highest when the soil was conditioned with *Oryza* while it was lowest when the soil was conditioned with *Triticum, Glycine, Zea* and *Lolium*. *Glycine* shoot biomass was significantly lower when grown in soils conditioned with *Glycine* itself and with *Medicago*. Shoot biomass of *Triticum* was lowest in soils conditioned with *Triticum*, *Zea* and *Lolium* and highest with *Lens*, *Medicago*, and *Trifolium*. Shoot biomass of *Zea* was the lowest in Glycine conditioned soil and the highest in *Medicago*, *Lens* and *Trifolium*. No significant differences were observed in shoot biomass of *Medicago* when grown in the eight conditioned soils.

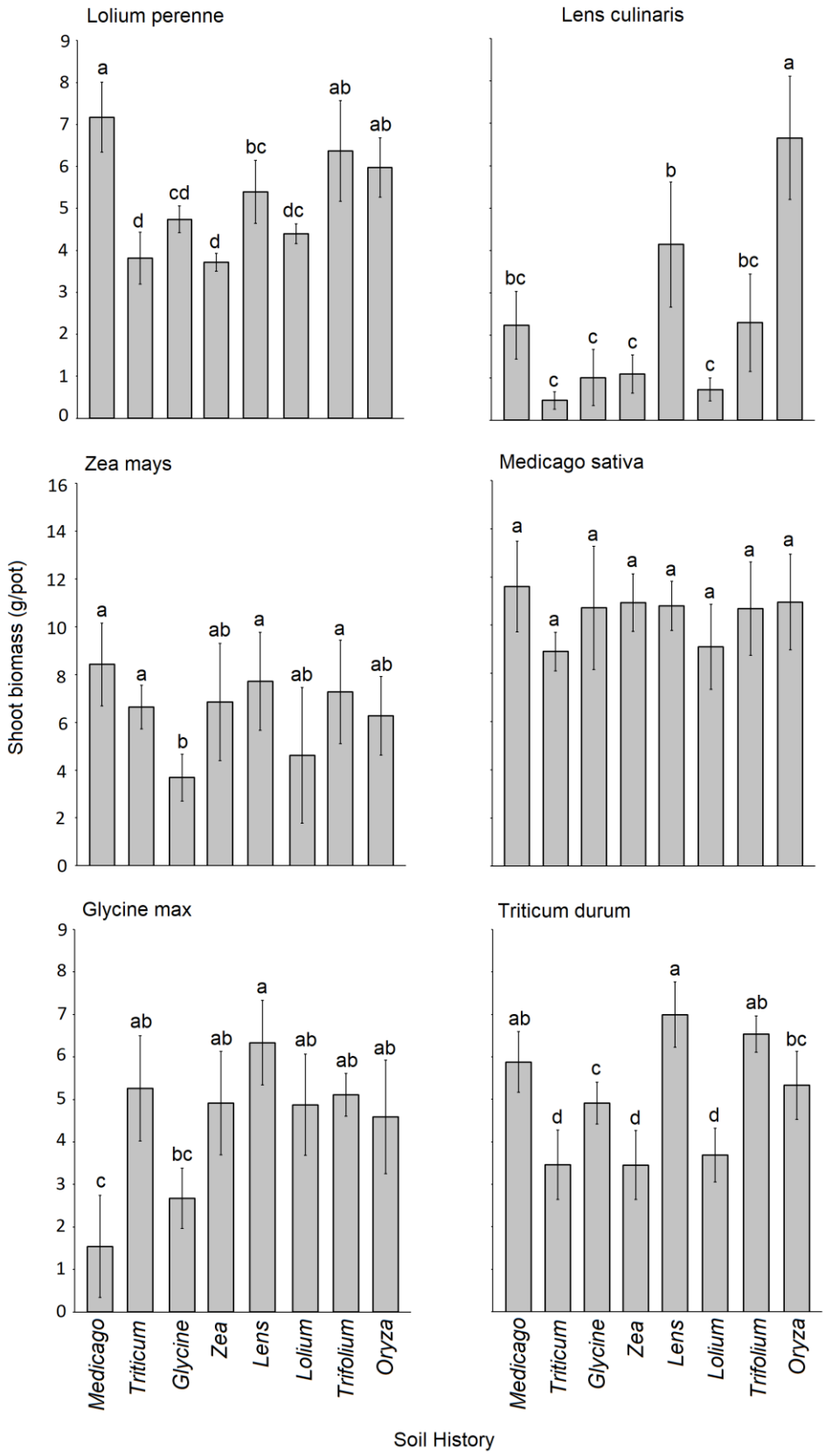

Fig 2. Average shoot biomass (g.pot-1) of each of the six response plants grown in each of the eight conditioning plant species' soil history. The error bars represent the standard deviation. Bars topped by the same letter do not differ significantly by Tukey *post hoc* test.

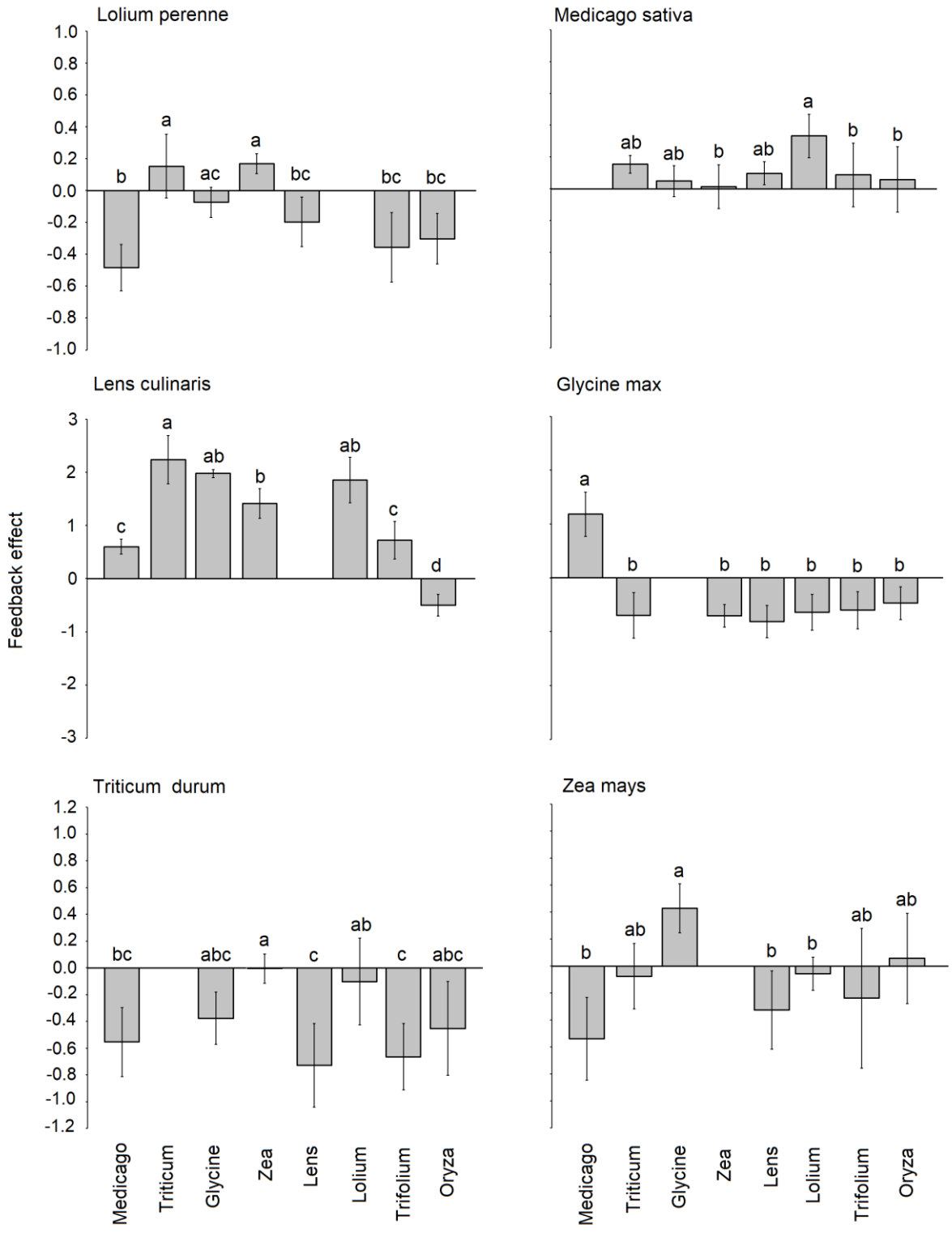

Fig 3. Feedback effect of each of the six response plants i.e. biomass of the each response plant grown in its own conspecific conditioned soil divided on the biomass of the same plant species grown on each of the heterospecific seven conditioned soils. The error bars represent the standard deviation. Bars topped by the same letter do not differ significantly by Tukey *post hoc* test.

6.4.2 Plant-soil feedback

Our results show that the tested plant species exhibited different feedback depending on the conditioned soil (Fig. 3). In details, significant negative feedback was produced for *Lolium* when grown on soil conditioned with *Medicago*, *Lens*, *Trifolium* and *Oryza*. In contrast, a significant positive feedback was generated when the soil was conditioned with *Triticum* and *Zea*. Similarly, *Zea* suffered significant negative feedback when grown on soil conditioned with *Medicago*, *Lens* and *Lolium*, while significant positive feedback occurred when grown on soil conditioned with *Glycine*. On the other hand, *Triticum* and *Glycine* showed strong negative feedback when grown in all conditioned soils, except for the response of *Glycine* in a conditioned *Medicago* soil, which showed strong positive feedback. *Lens* and *Medicago* showed significant positive feedbacks when grown in each of the eight conditioned soils, except for *Lens*, which showed a negative feedback effect when grown in conditioned *Oryza* soil. In general, the overall feedback effect showed that *Glycine* exhibited the strongest negative feedback followed by *Triticum*, while low feedback was recorded for *Lolium* (Fig. 4). On the other hand, *Lens* was the only crop that showed strong positive feedback, while slight positive feedback was recorded for both *Zea* and *Medicago*.

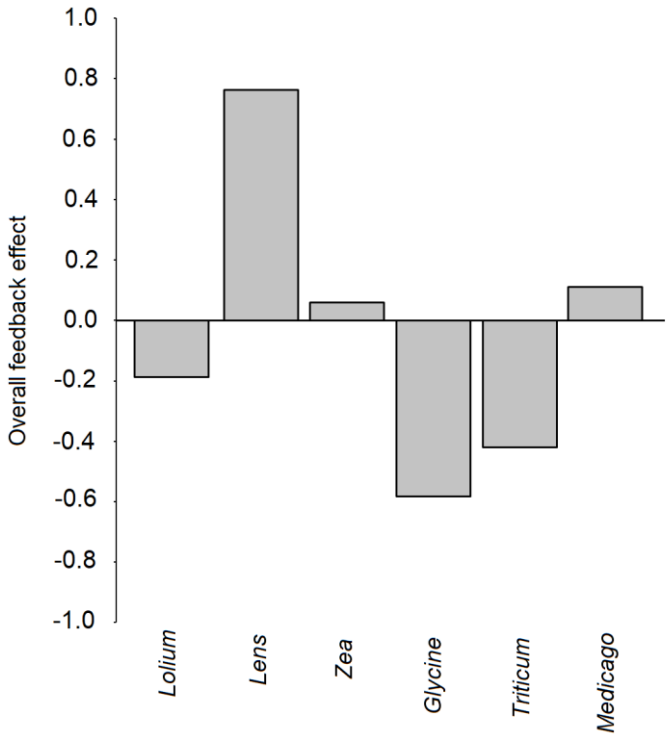

Fig 4. Overall feedback effect of the six response plant species grown in the eight conditioned soil histories.

6.4.3 Soil chemical properties and correlation with biomass

Soil chemical parameters showed significant differences among the conditioned soils (Table 1). In details, *Glycine* conditioned soil had significantly the highest content of total limestone, organic matter, total nitrogen, Mg and Fe compared to the other conditioned soils, while the content of Mn, Cu, Ca, K, P, Na, Cl and soil pH were significantly low compared to the other conditioned soils. Moreover, *Triticum* soil had significantly the highest content of chlorides, while *Lens* had the highest content of Zn compared to other soils. However, no significant differences were found between the soils in terms of electrical conductivity and soil sodium content.

Table 1. Chemical analysis of each of the eight soil histories.

	Zea	Lens	Oryza	Glycine	Triticum	Lolium	Trifolium	Medicago	p-value
pH	8.05a	8.09a	8.17a	5.83b	8.27a	8.22a	8.11a	8.17a	< **0.001**
Total limest (%)	1.67b	1.58b	1.73b	4.42a	2.38b	1.20b	1.91b	1.89b	< **0.001**
EC (mS/cm)	0.22a	0.22a	0.23a	0.21a	0.31a	0.23a	0.26a	0.20a	0.366
Cl (g/Kg)	0.07ab	0.06b	0.07ab	0.03b	0.20a	0.07ab	0.09ab	0.06ab	**0.039**
Na (g/Kg)	0.33ab	0.30ab	0.30ab	0.19b	0.38ab	0.50a	0.33ab	0.34ab	0.116
OM (%)	3.39c	3.41bc	3.30c	9.52a	3.33c	3.58bc	3.58bc	3.68b	< **0.001**
Total N (%)	0.19b	0.18b	0.18b	0.58a	0.19b	0.19b	0.18b	0.19b	< **0.001**
P (mg/Kg)	134.32a	136.58a	155.33a	55.15b	141.23a	130.91a	139.a	124.93a	**0.001**
K (g/kg)	2.08a	2.12a	2.12a	0.92b	2.28a	2.35a	2.11a	2.43a	< **0.001**
Mg (g/Kg)	0.55b	0.56b	0.57b	0.94a	0.57b	0.58b	0.56b	0.56b	< **0.001**
Ca (g/Kg)	6.34a	6.55a	6.66a	5.00b	6.63a	6.37a	6.26a	6.11a	**0.001**
Cu (mg/Kg)	40.95a	41.49a	41.71a	13.02b	39.65a	39.80a	39.80a	39.66a	< **0.001**
Zn (mg/Kg)	20.94ab	24.18a	21.62ab	20.46ab	19.49ab	18.90b	21.44ab	17.76b	**0.015**
Mn (mg/Kg)	31.02a	32.13a	33.19a	3.65b	26.33a	31.92a	33.31a	30.43a	< **0.001**
Fe (mg/Kg)	31.56b	31.65b	31.57b	103.27a	43.63ab	32.30b	30.81b	29.84b	**0.033**

Pearson correlation between shoot biomass and soil chemical parameters (Fig. S1) showed a strong significant positive correlation between Fe, Mn, Cu, Ca, Na, Cl, total N and $CaCO_3$ with *Glycine* biomass, while a strong negative correlation was found with soil pH. Moreover, *Lens* biomass was positively correlated with Zn and K content and negatively correlated with $CaCO_3$, while *Medicago* biomass was significantly correlated with soil P, EC and total N content. On the other hand, *Lolium* biomass showed significant positive correlation with Mn, Ca and K content while it was negatively correlated with total N content. In addition, the contents of Mn, Zn and Cu were negatively correlated with the growth of *Triticum,* while Ca and soil pH showed a positive correlation. Furthermore, biomass of *Zea* was negatively correlated with P, Ca, K, Na and soil pH, while it was positively correlated with soil Fe content.

6.4.4 Microbial diversity and community composition

Our results show that no significant differences were observed in the number of bacterial species, the number of ASVs and the Shannon diversity index (Fig. 5). On the other hand, the number of fungal species was significantly low in *Glycine* conditioned soil compared to *Triticum* and *Lolium*, while the number of ASVs was significantly low in *Glycine*-conditioned soil compared to *Triticum*, *Lens*, *Lolium* and *Trifolium*. In contrast, no significant change was observed in Shannon diversity index for fungi among the conditioned soils.

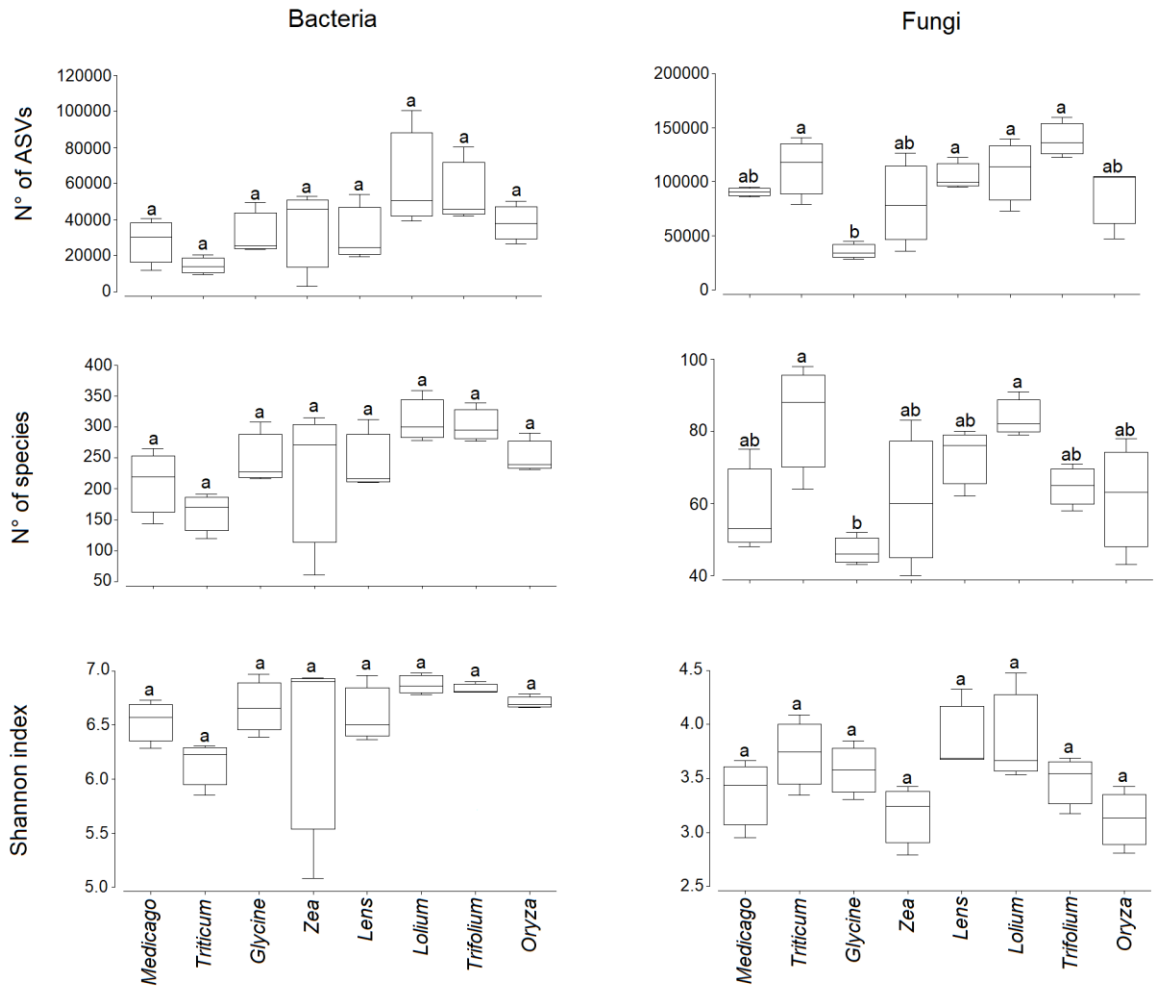

Fig 5. Box plots showing the variation in the Shannon diversity, number of species and reads for bacterial and fungal communities in the eight soil histories. Different letters indicate significant ($p < 0.05$) differences in the indices. The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median, whiskers above and below the boxplot indicate inter-quartile range.

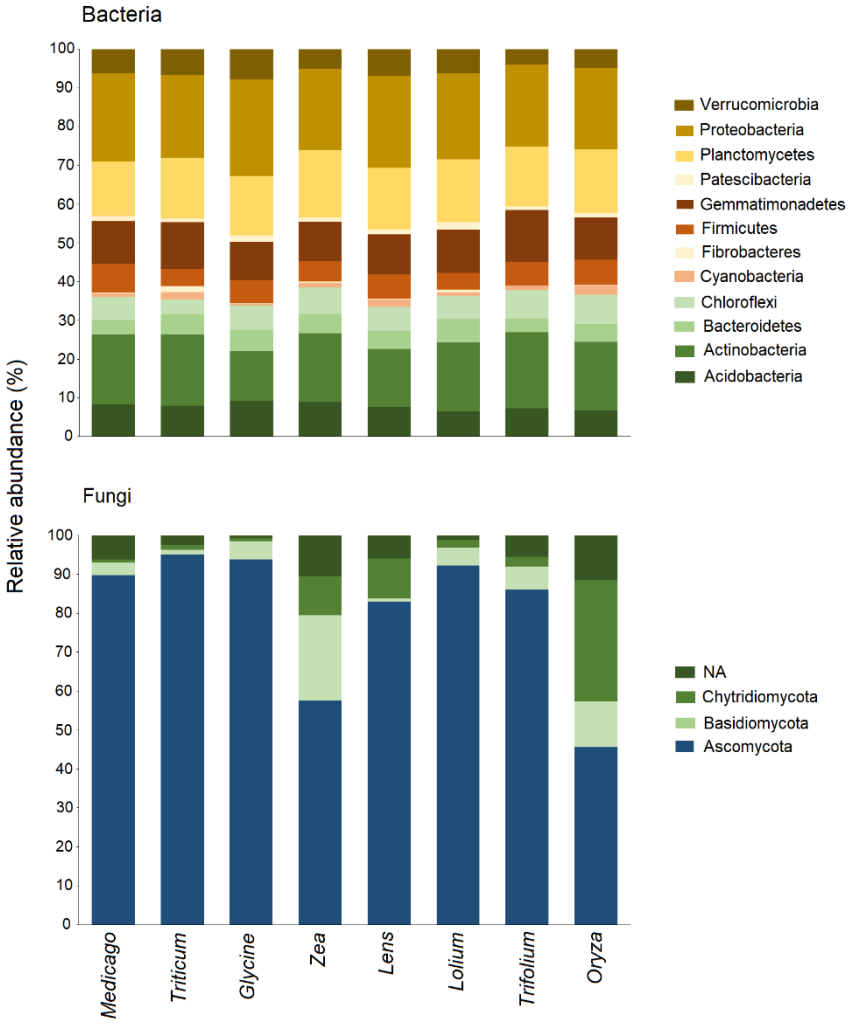

Fig 6. The relative abundance of various bacterial and fungal phyla in the soil of each soil history.

At the phylum level (Fig. 6), the bacterial community varied among the conditioned soils. In details, all conditioned soils contained mainly *Proteobacteria*, ranging from 20.6% in *Triticum* to 25.5% in *Glycine* soils. On the other hand, the highest percentage of *Actinobacteria* was found in *Trifolium* and *Triticum* soils with 19.3% and 18.0%, respectively, while the lowest abundance was recorded in *Glycine* soil with a percentage of 12.8%. *Planctomycetes* abundance, however, ranged from 14% in *Medicago* soil to 17.2% in *Triticum* soil. While *Gemmatimonadetes* were very abundant in *Trifolium* soil at 13.2% and less abundant in *Glycine* soil at 9.2%. Moreover, the highest levels of *Acidobacteria* were found in *Glycine* soil (9.2%) followed by *Zea* soil (8.9%) while the lowest levels were found in *Lolium* (6.4%) and *Oryza* (6.7%) soils. *Verrucomicrobia*, on the other hand, was most abundant in *Glycine* soil (7.4%) and less abundant in *Trifolium* soil (3.9%). Nevertheless, the fungal community showed a clear variation among the conditioned soils (Fig. 6). In particular, all the soils studied were dominated by the phylum *Ascomycota*, with abundance ranging from 95.1%, 93.9% and 92.3%

in the soils of *Triticum*, *Glycine* and *Lolium*, respectively, to 45.6% and 57.6% in the soils of *Oryza* and *Zea*, respectively. However, the highest percentage of the phylum *Basidiomycota* was found in the soil of *Zea* (21.9%), followed by *Oryza* (11.7%), while their abundance did not exceed 5% in the other soils. On the other hand, the phylum *Chytridiomycota* was found in *Oryza* soil with an abundance of 31.2%, followed by *Lens* (10.3%), *Zea* (10%) soils, and less than 2% in the other soils.

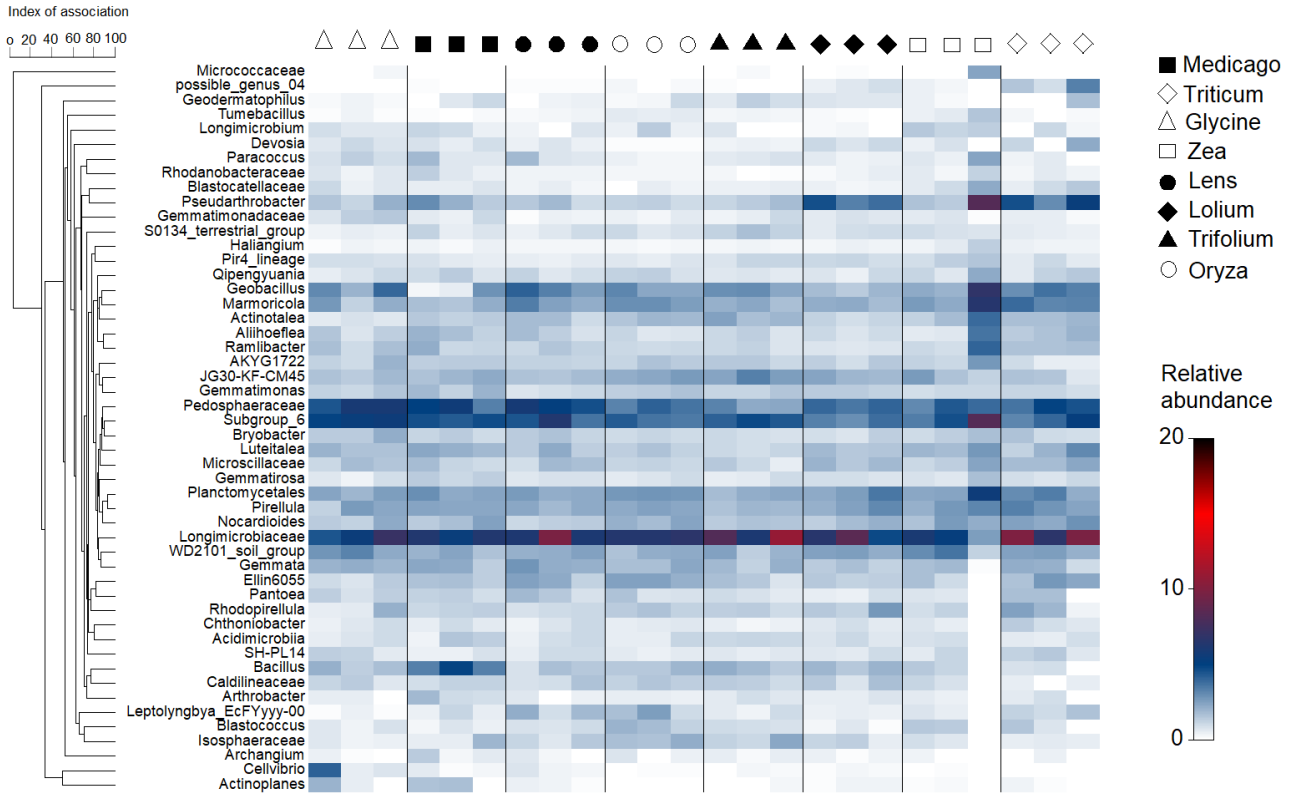

Fig 7. Heatmap showing relative abundance of the 50 most frequent Amplicon Sequence Variants (ASVs) in the bacterial community in each of the eight soil histories. The hierarchical grouping of variables is based on Whittaker's association index.

At low taxonomic level, the bacterial heatmap showed a slight difference between the conditioned soils with respect to the 50 most common ASVs (Fig. 7). Specifically, we found that all conditioned soils had high abundance of *Acidobacteria* subgroup_6 and *Longimicrobiaceae*, while *Pedosphaeraceae* were more abundant in *Glycine*, *Medicago* and *Lens* than in the other soils. In addition, a large amount of *Pseudarthrobacter* was found in *Lolium*, *Zea* and *Triticum* soils, while *Bacillus* was more abundant in *Medicago* soil. On the other hand, the fungal heatmap showed a clear variation among the conditioned soils with respect to the 50 most abundant ASVs (Fig. 8). In particular, *Plectosphaerella cucumerina* was more abundant in *Lolium* and *Triticum* soils, while *Plectosphaerella oratosquillae* was highly

abundant in conditioned *Triticum* soil. *Botryotrichum atrogriseum*, on the other hand, was particularly abundant in *Glycine* soil, followed by *Oryza*. In addition, *Fusarium solani* was exclusively found in *Glycine* soil followed by *Triticum*. *Zea* soil, however, contained the highest abundance of *Fusarium acutatum*, followed by *Triticum*. Moreover, *Paramyrothecium foliicola* was highly abundant in *Lolium*, *Medicago* and *Trifolium* soils, while *Stachybotrys chartarum* was most abundant in *Lolium* and *Trifolium* soils. Furthermore, *Alternaria* and *Cladosporium delicatulum* were very common in *Medicago*, *Trifolium* and *Lens* soils. *Stemphylium*, on the other hand, was highly abundant only in *Medicago* soil and less abundant in *Lens* soil.

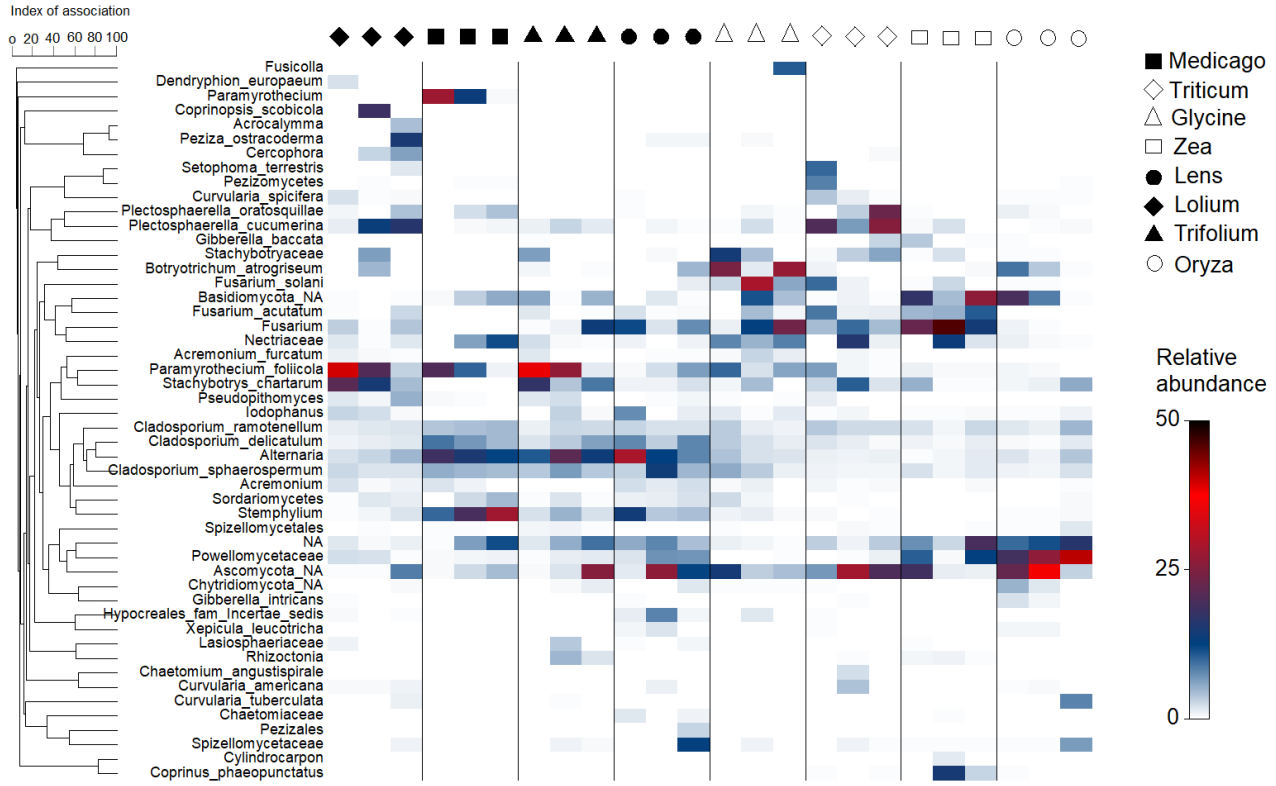

Fig 8. Heatmap showing relative abundance of the 50 most frequent Amplicon Sequence Variants (ASVs) in the fungal community in each of the eight soil histories. The hierarchical grouping of variables is based on Whittaker's association index.

6.4.5 Linking microbial community to soil chemical properties

The nMDS analysis of the bacterial community in terms of chemical parameters (Fig. 9) showed that the ordination of *Medicago*, *Glycine* and *Lens* samples was strongly correlated with soil organic matter content, while the ordination of *Lolium*, *Oryza*, *Trifolium* and *Zea* samples was positively correlated with Mn and Zn content. However, P content showed positive correlation with ordination of *Triticum* samples. The other soil chemical parameters

showed negative correlation with bacterial ordination of all soil samples. As for the ordination of samples based on fungal community, nMDS analysis showed that Cu, Mn, K, pH, Na, $CaCO_3$, EC, P, total N and OM were positively correlated with soil samples of *Medicago*, *Lens*, *Lolium* and *Trifolium*. Whilst ordination based on fungal community of *Triticum*, *Glycine*, *Zea* and *Oryza* was positive and strongly correlated with Cl content, followed by Zn, Mg, Fe and Ca contents.

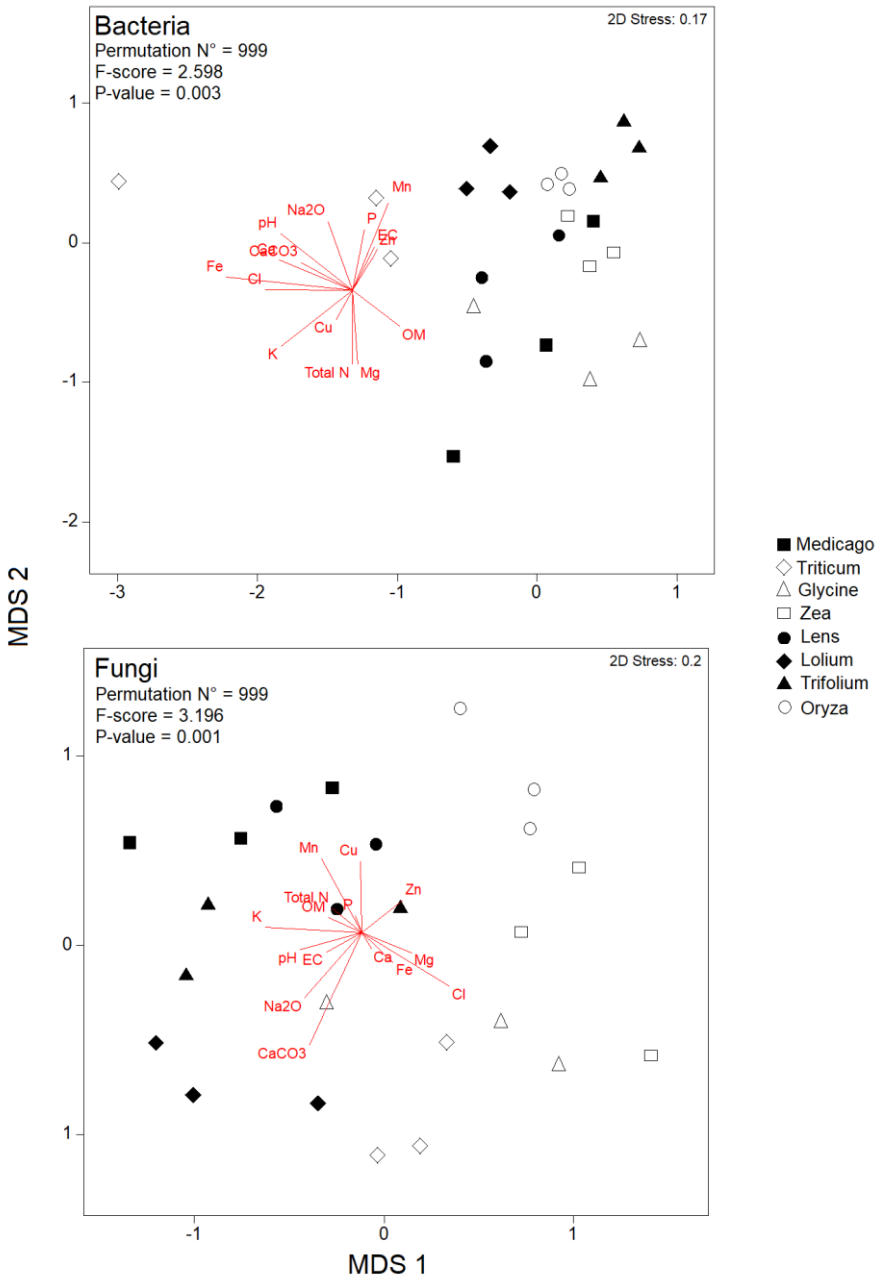

Fig 9. Nonmetric multidimensional scaling (NMDS) plots of bacteria and fungi communities in each of the eight soil histories. MDS axis 1 and MDS axis 2 represent the two axes of the two-dimensional ordination space. Each point represents the microbiome of one replicate of the plant. The stress-level

shown in each plot indicates how well the individual distances between objects are represented (between 0 and 1; the closer to 0, the better are original data points represented in the ordination space). Vectors represent soil environmental variables that significantly correlated with the ordination ($p < 0.05$ based on 999 permutations).

6.4.6 Linking microbial community to crop response

We constructed six co-occurrence networks (Fig. 10) and calculated five topological parameters to assess interactions among ASVs and with the response biomass for each of the five networks (Table S1). The number of nodes ranged among the networks from 77 in *Medicago* to 91 in *Lolium*, whereas the number of edges ranged from 1022 in the *Medicago* network to 1809 in *Lens*. The percentage of positive ASVs correlations in the microbial networks ranged from 57.6% in *Zea* to 78.7% in *Lolium*. The network diameter varied among soils, from the lowest value of 5 in *Lens* and *Medicago* to the highest value of 7 in *Glycine* network. Moreover, the network density was highest in *Zea* and *Lens*, while it was lowest in *Glycine*. However, the values of characteristic path length and clustering coefficient showed no significant changes among the conditioned soil networks. On the other hand, modularity recorded the highest value of 1.537 in *Zea* network while the lowest values of 0.339 and 0.452 were recorded in *Lens* and *Lolium* soils, respectively.

The co-occurrence network showed that *Glycine* biomass had significantly strong negative interactions with *Arthrobacter*, *Bacillus*, *Caldilineaceae*, *Longimicrobium*, *Powellomycetaceae* and *SH _PL14* ASVs, while *Lens* biomass was negatively correlated with *Acremonium*, *Actinoplanes*, *Chaetomiaceae*, *Cladosporium delicatulum*, *Fusarium* and *Nectriaceae* ASVs. On the other hand, *Lolium* biomass had strong negative interactions with *Acrocalymma*, *Curvularia tuberculata*, *Fusarium acutatum*, *Fusarium solani*, *Plectosphaerella cucumerina*, *Setophoma terrestris* and *Stemphylium* ASVs. *Triticum* however showed significant negative interactions with ASVs *Alternaria*, *Cladosporium ramotenellum*, *Gibberella baccata*, *Paramyrothecium* and *Plectosphaerella oratosquilla* while the ASVs that showed significant negative interactions with *Medicago* biomass are *Acremonium*, *Alternaria*, *Cellvibrio*, *Cladosporium delicatulum*, *Cladosporium sphaerospermum*, *Curvularia Americana*, *Devosia*, *Fusarium acutatum*, *Geobacillus*, *Haliangium*, *Paramyrothecium foliicola*, *Paracoccus*, and *Tumebacillus*. Finally, *Zea* biomass showed significant negative interactions with the following ASVs: *Acremonium*, *Cladosporium delicatulum*, *Cladosporium ramotenellum*, *Fusarium acutatum*, *Iodophanus*, and *Stemphylium*.

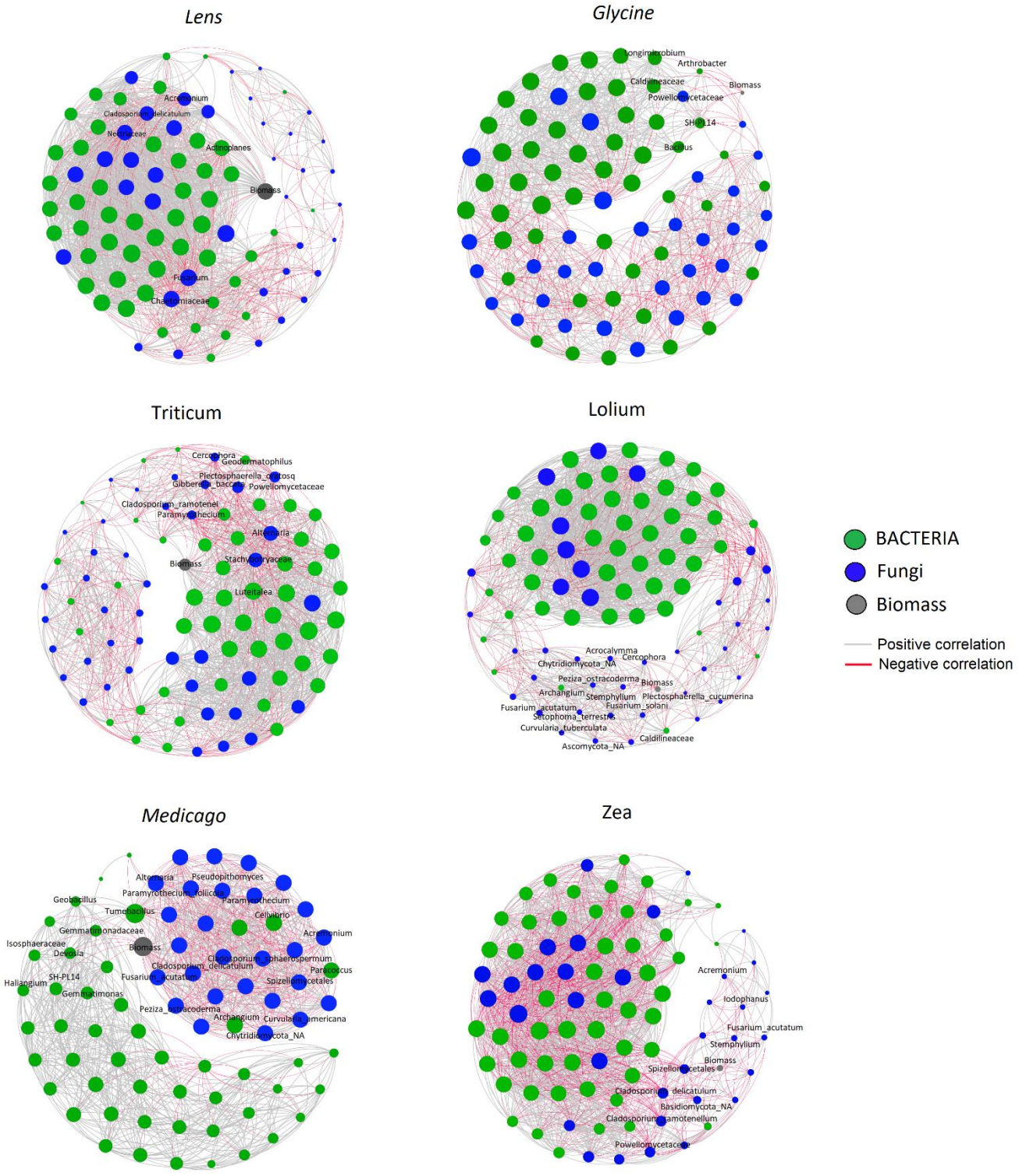

Fig 10. Correlation network analysis showing potential interactions between bacterial, fungal families and response biomass in conspecific soil for each of the six response plants. The lines connecting nodes (edges) represent positive (grey) or negative (red) co-occurrence relationship. The connection stands for a strong (Spearman's $\rho>0.6$ and $\rho<-0.6$) and significant (p-value<0.05) correlation. The size of each node is proportional to the ASV relative abundance, only the top 50 ASVs were kept. The nodes were coloured by kingdom level.

6.5 Discussion

In this study, we compared the legacies of eight plant species, belonging to grass and nitrogen-fixing functional groups, on conspecific and heterospecific plant performances, soil chemical properties, and soil microbiota using a PSF approach. Overall, we found that plant species-specific legacies affect all of these variables in some way. Indeed, our study has shown that response plant species exhibit different feedback behaviours depending on the previously conditioned soil. Previous studies have shown that the direction and effect sizes of PSF appear to differ between functional plant groups (Kulmatiski et al. 2008; Meisner et al. 2014). However, our study shows that PSF may or may not be specific to functional groups. We observed that the grass *Lolium* produces a strong negative feedback when grown in soils conditioned by the legumes *Medicago, Lens, Trifolium*, and the grass *Oryza* (i.e., lower growth of *Lolium* in soils conditioned by *Lolium* compared to growth of *Lolium* in soils conditioned by *Medicago, Lens, Trifolium*, and *Oryza*), whereas it showed a strong positive feedback when grown in soils previously conditioned by the grasses *Triticum* and *Zea* (i.e., higher growth of *Lolium* in soils conditioned by *Lolium* compared to growth of *Lolium* in soils conditioned by *Triticum* and *Zea*). Moreover, *Zea* grass suffered a significant negative feedback when grown in soils conditioned with the legumes *Medicago, Lens* and the grass *Lolium*, while a significant positive feedback occurred when grown in soil conditioned with *Glycine* legume. Notably, *Glycine* showed the strongest negative feedback in its own soil and negative feedback when grown in all conditioned soils except *Medicago* legume soil where it showed strong positive feedback. On the other hand, the grass *Triticum* suffered from a strong negative feedback in all conditioned soils without exception. Similarly, Hannula et al. (2019) concluded that the direction of the PSF could not be predicted solely from the plant group or family, even though soils from grasses tended to have more positive feedbacks than soils conditioned by forbs and legumes.

The observed patterns in the present study may be driven by two mechanisms, i.e., soil nutrient availability and/or soil microbial communities. The advantage of using the 'conspecific' and 'heterospecific' feedback approach is that it can shed light on the chemical legacies produced by the decomposition of litter and root exudates of different plant species during the conditioning phase. Originally, we assumed that each plant species would alter the chemical properties of the soil in a unique way and that these changes could affect subsequent growth. However, our results show that the differences in the chemical legacies produced by the eight plant species were statistically insignificant, with the exception of *Glycine*. Soil

conditioned by *Glycine* had the highest content of total limestone, organic matter, total nitrogen, Mg and Fe compared to the other conditioned soils, while the content of Mn, Cu, Ca, K, P, Na, Cl and soil pH were significantly low. Legumes live in symbiosis with nitrogen fixing rhizobacteria and we hypothesize that the feedback effects would be due to nitrogen availability, even though we did not detect differences in nitrogen availability in the soil chemical analysis between legumes and grasses except in *Glycine* conditioned soil. However, we observed that *Glycine*, which had the strongest negative feedback effect, had the highest nutrient pool, supporting the fact that priority effects in plant communities are not just a matter of resources availability. Since *Triticum* and *Lolium*, which also suffered from a strong negative feedback effect, did not differ in terms of chemicals with *Lens*, which had the highest positive feedback effect, we could speculate that the diversity of conditioning plant species has little effect on subsequent plant growth by altering nutrient availability in the soil. Similarly, a recent study by Xue et al. (2021) showed that differences in soil chemical analysis between soils conditioned by four grasses, including *Lolium perenne*, and three legumes, including *Medicago Sativa* and *Trifolium repens*, had no effects on subsequent plant invasion in a plant-soil feedback experiment.

Alternatively, soil microbes are thought to significantly influence PSF, both directly by affecting plant growth or defence responses, and indirectly, for example, by affecting mineralization or acting as antagonists of plant pathogens (van der Putten et al. 2013; Chialva et al. 2018). Our results showed that microbial diversity, i.e. Shannon diversity index, did not show significant differences among the eight conditioned soils. Moreover, the effect of conditioning on microbial community composition showed no specificity between the two plant functional groups. However, the abundance of functionally important microbial phyla was affected by plant legacies. We found that *Actinobacteria* were highly abundant in the soils of legume *Trifolium* and grass *Triticum*, while less abundant in the soils of legume *Glycine*. Moreover, *Gemmatimonadetes* were very abundant in *Trifolium* soils and less abundant in *Glycine* soils. Similarly, the highest levels of *Acidobacteria* were found in *Glycine* soils followed by *Zea* soils while the lowest levels were found in *Lolium* and *Oryza* soils. *Verrucomicrobia*, on the other hand, was most abundant in *Glycine* soils and less abundant in *Trifolium* soils. Moreover, *Ascomycota* abundance was the highest in *Triticum* and *Glycine* soils, and the lowest in *Oryza* soils. However, the phylum *Basidiomycota* was mostly found in the soil of *Zea* followed by *Oryza*. Our results indicate that each plant species, rather than plant functional groups, generates its own microbial legacy in the soil after a period of conditioning.

In contrast, several recent works have shown that plant family and functional group can explain a large portion of the variation in microbial community structure (Dassen et al. 2017; Hannula et al. 2021). Moreover, Connell et al. (2021) found a significant legacy of conditioning by *Bromus inermis* that affected not only the bacterial community composition, but diversity as well. We hypothesised that microbial community composition would be altered by individual plant species, possibly due to the higher amount and/or diversity of litter that falls, decomposes and enters the soil C and N cycles (Hooper et al., 2000). Previous studies suggest that litter decomposition in soil can alter microbial biomass, composition and community structure by increasing substrate variability and diversity of soil chemical compounds, and that this can vary depending on the plant species (Chapman et al., 2013). Indeed, *Glycine* soils with the highest content of total limestone, organic matter, total nitrogen, Mg and Fe and the lowest content of Mn, Cu, Ca, K, P, Na, Cl and soil pH have been shown to have the lowest abundance of *Actinobacteria*, *Gemmatimonadetes* and *Ascomycota*, while the abundance of *Acidobacteria* and *Verrucomicrobia* is the highest. These results were confirmed by our nMDS analysis showing that the ordination of the microbial community for each plant species was strongly correlated with different soil chemical parameters.

Interestingly, our results show that at the low taxonomic level, the bacterial ASVs *Pedosphaeraceae* were more abundant in the legumes *Glycine*, *Medicago*, and *Lens* soils, while *Bacillus* was more abundant in *Medicago* soils. A recent study by Yuan et al. (2022) showed the effect of *Pedosphaeraceae* as key bacteria that have significant potential as plant growth promoting rhizobacteria with interspecific interactions for phytoremediation. In addition, *Bacillus* species are a major type of rhizobacteria that can form spores that can survive in soil for a long time under harsh environmental conditions (Hashem et al. 2019). Indeed, their high abundance in the soil of legumes is due to their exclusive symbiotic ability. Moreover, our results show that a large amount of *Pseudarthrobacter* was found in the soils of *Lolium*, *Zea* and *Triticum* grasses. It has been reported that *Pseudarthrobacter* are a group of endophytic bacteria that can be isolated from soils, deserts, and mines (Finger et al. 2019; Chai et al. 2019), and that *Pseudarthrobacter sulfonivorans* strain Ar51 can efficiently degrade petroleum and several benzene compounds at low temperatures (Zhang et al. 2016). Previous studies have shown that the effects of soil legacy can be explained mainly by soil fungal composition (Bezemer et al., 2006; Wang et al., 2019). However, our results show that the abundance of specific fungal species or groups of fungi in the soil is much more important for plant growth and thus PSF than the composition of the entire fungal community. Interestingly, we found that

the soil of *Glycine* contained high abundance of *Botryotrichum atrogriseum* and *Fusarium solani*, while the soil of *Triticum* contained high abundance of *Plectosphaerella cucumerina*, *Plectosphaerella oratosquillae*, *Fusarium solani* and *Fusarium acutatum*. On the other hand, the soil of *Lolium* contained high relative abundance of *Plectosphaerella cucumerina*, *Paramyrothecium foliicola* and *Stachybotrys chartarum*. *Lens* soil, however, contained high levels of *Alternaria* and *Cladosporium delicatulum*, while *Medicago* soil had in addition high levels of *Stemphylium*. Our results indicate that each plant species conditioned its own soil with a high proportion of putative pathogenic fungi, which could explain the direction of the generated PSF in the response phase. However, when linking the conspecific biomass to different ASVs present in the soil, our co-occurrence analysis showed that all plant species had a strong significant negative correlation with fungal pathogens accumulated in the soil, with the exception of *Glycine*, which showed a strong negative correlation with plant growth-promoting rhizobacteria such as *Arthrobacter* and *Bacillus*, suggesting that the ability to predict PSFs requires better understanding of plant interactions with diverse communities of plant pathogens and mutualists, rather than single host-specific pathogens (Bever et al. 2012; Benítez et al. 2013).

6.6 Conclusion

Our study show, in contrast with many previous works, shows that response plant species exhibit different feedback behaviours, but independently of plant functional groups. Originally, we assumed that each plant species altered soil chemical properties in a unique way and that these changes could influence subsequent growth. However, our results indicate that differences in chemical legacies among the eight plant species were statistically insignificant, with the exception of *Glycine*. However, we observed that *Glycine*, which exhibited the strongest negative feedback effect, had the highest nutrient pool, supporting the fact that priority effects in plant communities are not simply a matter of competition for shared resources. Furthermore, our results indicate that microbial diversity did not show significant differences in the eight conditioned soils. Moreover, the effect of conditioning on microbial community composition showed no specificity between the two plant functional groups. However, the abundance of functionally important microbial phyla was affected by plant legacies. Therefore, we can assume that each plant species, rather than the plant functional groups, generates its own soil microbial legacies. Previous studies have shown that the effects of soil legacies can be explained mainly by soil fungal composition. However, our results show that the abundance of specific fungal species or fungal groups in the soil is much more

important for plant growth, and thus PSF, than the composition of the entire fungal community. Finally, our results suggest that each plant species was associated with a certain proportion of fungal pathogens. However, when linking the conspecific biomass to different fungal species present in the soil, we showed that all plant species had a strong significant negative correlation with fungal pathogens accumulated in the soil, with the exception of *Glycine*, which instead had a strong negative correlation with plant growth-promoting rhizobacteria, suggesting that the ability to predict PSFs requires a better understanding of plant interactions with different communities of plant pathogens and mutualists, rather than with specific pathogens.

7 Chapter 7: what drives plant-soil feedback in Arabidopsis thaliana: soil microbiota, chemical traits, or extracellular self-DNA?

7.1 Abstract

Plant-soil feedback processes (PSF) influence species coexistence and increase diversity when conspecifics are disadvantaged in their own soil relative to heterospecifcs. Evidences suggest nutrients depletion, natural enemies, or phytotoxicity as possible mechanisms to explain species-specific negative PSF. Moreover, a recent study has shown that extracellular self-DNA (self-exDNA) released during decomposition of plant litter could be a possible factor for litter autotoxicity and thus negative PSF. For this reason, we grew *Arabidopsis thaliana* L. to condition a living soil. After the conditioning phase, the soil was subjected to four different treatments, namely sterilization by autoclaving, washing under tap water, addition of 10% activated carbon and untreated control. After the treatments, the same species was sown as response plants. After the response phase, we assessed soil chemical properties and the microbiota by shotgun sequencing. In addition, we quantified for the first time the self-exDNA of *A. thaliana* in each of the treated soils as well as in the preconditioned soil using *chloroplast* rbcL DNA primers. Our results show that the highest biomass was obtained when the conditioned soil was sterilized, followed by the washing treatment, while the addition of activated carbon was the lowest and showed no significant difference compared to the control. Conversely, the highest concentration of self-exDNA was recorded in the soil treated with activated carbon, followed by the control, while sterilized and washed soils had the lowest concentration. Moreover, our results suggest that the direction and strength of the feedback between treatments is not due to the chemical properties of the soil, as we found that the soil with activated carbon, which suffered from a strong negative feedback effect, had the highest organic carbon content and did not differ in terms of P content from the washed soil, which showed a strong positive feedback effect. In addition, we found that the abundance of functionally important microbial phyla was affected by the treatments, especially sterilization, and that fungal pathogens were found in soils where growth was extremely promoted, i.e., sterilized and washed soils. Finally, our study provided evidence for the hypothesis that self-exDNA has an inhibitory effect on conspecifics and suggests that weakening a plant as a result of exposure to self-exDNA may ultimately increase its susceptibility to pathogen attack, thus enhancing the species-specific negative PSF.

Keywords: plant-soil feedback, autotoxicity, conspecifics, *Arabidopsis thaliana*, Shotgun sequencing, extracellular self-DNA

7.2 Introduction

Plant-soil feedback (PSF) describes the relative growth of a plant in its own conspecific soil compared to a heterospecific soil conditioned by a different plant species (Bever et al. 1997; Ehrenfeld et al. 2005). As plants grow, they alter the biotic and abiotic properties of the soil, which in turn affects the growth and survival of subsequent plants (Bever et al. 1997). Thus, plant responses to PSF can be negative, mainly through resource depletion, excretion of autotoxic compounds or accumulation of natural enemies, or positive through promotion of symbionts and/or availability of nutrients in the soil (Klironomos, 2002; Bever, 2003; van der Putten et al. 2013). Therefore, PSFs influence species coexistence and increase diversity when conspecifics are disadvantaged in their own soil relative to heterospecifcs (Bever, 2003; Bagchi et al. 2010; Crawford et al. 2019).

There are numerous data in the literature on the possible mechanisms underlying species-specific PSF (Kulmatisky et al. 2008; van der Putten et al. 2013), such as soil nutrient depletion (Ehrenfeld et al. 2005) and accumulation and/or altered composition of soil-borne pathogens (Packer & Clay, 2000; Kardol et al. 2007). Nutrient depletion generally causes negative PSF by limiting plant growth, while pathogens cause negative PSF by reducing plant performance at multiple life stages, although they tend to affect young susceptible plants (Sarmiento et al. 2017; Mommer et al. 2018). However, observations of long-term negative PSF in agriculture and natural ecosystems (Singh et al. 1999, Bonanomi et al. 2011) are examples of species-specific inhibitions that are not related to either nutrient availability or soil-borne pathogens. On the other hand, previous works have attempted to propose the inhibitory effect of litter phytotoxicity as another possible mechanism to explain species-specific negative PSF (Singh et al. 1999). The most common phytotoxic compounds released during the decomposition of litter in soil include short-chain organic acids such as propionic and butyric acids (Armstrong & Armstrong, 2001), tannins (Kraus et al. 2003) and low molecular weight phenols (Li et al. 2010). However, after their release into the soil, allelochemicals are affected by the soil, for example, through absorption, migration or decomposition by microorganisms and enzymes. The studies by Yenish et al. (1995) and Blum (1998) have shown that phenolic allelochemicals can be decomposed by soil microbes, and thus they do not reach active concentrations. Therefore, the rapid degradation of such toxins by soil microbial activity, in addition to their lack of specificity, precludes the possibility that they cause species-specific negative PSF.

However, many previous studies have pointed to an exception to the general pattern of inhibitory effects, namely long-lasting species-specific toxicity exhibited by litter released from conspecifics. Recently, Mazzoleni et al. (2015) demonstrated that self-DNA inhibitory effects could explain the autotoxicity of litter and probably contribute to the negative PSF, as this extracellular DNA (exDNA), which accumulates in both decayed litter and soil, was able to exert significant species-specific inhibitory effects despite the extent of its fragmentation by decomposition processes. The authors hypothesized that this inhibition effect represents a mechanism of maintaining diversity. Furthermore, Bonanomi et al. (2022, under review) presented for the first time, under field conditions, a novel method demonstrating that self-DNA, but not heterologous one, exerts acute toxic effects on *Alnus glutinosa* L. roots in a closed system. In contrast, Veresoglou et al. (2015) discussed that self-DNA in soil may act as a stress-signaling molecule for conspecifics rather than an inhibitory substrate, whilst Duran-Flores & Heil (2015) argued that self-DNA might belong to the group of damage-associated molecular patterns (DAMP) that cause the local development of resistance-related responses by the affected plant.

The persistence of exDNA is influenced by the soil environment, i.e., the chemical, physical, and biological properties of the soil. DNA persists in soil by adsorbing to soil minerals, humic substances, and organo-mineral complexes (Levy-Booth et al. 2007). Once bound to these particles, exDNA is partially physically protected from degradation by microbial DNases and nucleases, allowing it to persist for years (Agnelli et al. 2007; Nielsen et al. 2007). Such persistence ability combined with toxicity function confirms the long-lasting negative PSF occurrence in soil for both natural and agroecosystems (Miller, 1996; Hawkes et al. 2013). DNA persistence and degradation are the main processes that complete the DNA cycle in soil. Common sterilization methods such as autoclaving are also known to affect DNA molecules (Gefrides et al. 2010). Indeed, sterilization of soils has been reported to reduce negative PSF (Packer & Clay, 2000; Klironomos, 2002; Kardol et al. 2007). Furthermore, Mazzoleni et al. (2015) suggested that weakening a plant through exposure to extracellular self-DNA could ultimately increase its susceptibility to pathogen attack, which would explain the associated negative PSF. However, Wen et al. (2009) reported that exDNA is a component of root cap mucilage involved in the increased resistance of growing root caps to soil-borne pathogens, and that degradation of exDNA leads to its loss. In this context, we conditioned *Arabidopsis thaliana* L. over a period of six months to affect soil biotic and abiotic properties through both root exudates and litter decomposition. After the conditioning period, the plants

were removed and the soil was subjected to four different treatments, namely sterilization by autoclaving, washing with tap water, addition of 10% activated carbon and untreated control. After the treatments, the same species was sown as response plants. After the response phase, the plant biomass grown in each of the four treatment was recorded, as well as soil chemical properties and microbiota using Shotgun sequencing. In addition, we quantified for the first time the self-DNA of *A. thaliana* in each of the treated soils as well as in the preconditioned one using *chloroplast* rbcL DNA primers. We expect that *A. thaliana* will affect soil chemical properties and biotic composition during the conditioning phase and that the amount of self-DNA will increase after conditioning. Moreover, we expected that the treatments applied would reduce the negative PSF effects due to their known functions in the soil. The specific objectives were:

i. to prove the occurrence of negative PSF for *A. thaliana* after a long-term conditioning period.

ii. to demonstrate that *A. thaliana* altered soil chemical and microbial, and functional properties.

iii. to provide evidence for the accumulation of self-DNA in the soil after conditioning.

iii. to demonstrate that soil sterilization, washing and activated carbon addition helped in reducing the intensity of the negative PSF whether by changing the chemistry, the microbiota, or by affecting the amount of self-DNA in the soil.

7.3 Material & Methods

The experiment was divided into two phases: the conditioning and the response phase. The target species used in this experiment, *A. thaliana*, was selected for its important characteristics, which include a short life cycle, a small size that limits growth requirements, seed production through self-pollination, and a strong ability to grow in the laboratory. The seeds used in this experiment were collected after growing commercially purchased seeds without prior treatment (De Corato sementi^{R}) for three succession cycles.

The soil used for this experiment was collected, in September 2018, in Parco Gussone within the Faculty of Agriculture (Federico II University of Naples, Italy) at a depth of 10 cm. The soil was immediately taken to the laboratory of the same faculty, manually homogenized

and sieved (< 0.5 cm) to remove coarse fragments. The soil was then sterilized by autoclaving (saturated high-pressure steam at 121 °C for 20 min) three times with 24 h interval, and then used for the conditioning phase. The soil had the following properties: 22.1% clay, 56.6% silt, 21.3% sand, pH 7.74, electrical conductivity 0.32 dS m^{-1}, organic carbon 15.4 g kg^{-1}, total nitrogen 1.6 g kg^{-1}, C/N ratio 9.6, $CaCO_3$ 7.16 g kg^{-1}, available phosphorus 239 mg kg^{-1}.

7.3.1 Soil conditioning phase

In this phase, the plants are cultivated in the soil for a certain period to condition it and to change the local biotic and abiotic conditions (Ehrenfeld et al. 2005; Van der Putten et al. 2013).

In early October 2018, *A. thaliana* seeds were sown in small pots (20 cm opening diameter ∗ 20 cm height ∗ 15 cm base diameter), each filled with 500 g of sterilized soil, with fifteen replicates totaling fifteen pots at this phase. Twenty seeds were sown in each of the replicate pots. Seeds were surface sterilized in a 3% sodium hypochlorite solution for 1 minute and rinsed several times with sterile water before use. All pots were irrigated with deionized water three times a week to maintain a field capacity above 50%. Shading, water and light stress were avoided during the experiment. Plants were grown for three months at a day length of twelve hours.

At the end of the conditioning phase, shoots were cut off at the soil surface. The soil from the replicate pots of the species was mixed and homogenized together with the roots in a container and then divided into four parts. Three of these parts were designated for three different treatments and one was kept as a control. One part of the soil was sterilized by autoclaving at 121 °C for 20 min. To the second part of the soil, 10% commercial activated carbon was added and mixed well to homogenize it, and the third part was put into 100 denier stockings and then washed under running tap water for 4 days. These soils were used as conditioned soils in the feedback phase after treatment.

7.3.2 Soil response phase

After the soil treatments, plastic pots (3 cm diameter * 2 cm depth) were each filled with 20 g of the treated conditioned soil. The pots were sterilized with 0.05% sodium hypochlorite solution before the experiment.

On April 2019, ten seeds of *A. thaliana* were sown in each of the response pots. Thus, 360 plants were included in this phase (ten seeds per pot * four treatments * nine replicates for each treatment). The pots were kept under a light chamber and irrigated three times a week with

deionized water to field capacity. After three months, all plants were harvested. Shoots and roots were washed to remove soil residues and dried at 70 °C for 72 h and their dry weight was recorded. For each treatment, soil from all replicate pots was mixed and then divided into three fractions: one fraction was stored at 4 °C to study the chemical properties of the soil, one fraction was stored at -20 °C and used for Shotgun sequencing, and the remaining fraction was stored at -20 °C and used to quantify the extracellular self- DNA of *A. thaliana*.

7.3.3 Soil chemical analysis

At the end of the experiment, soil samples destined for chemical analysis were dried in a ventilated chamber at room temperature until a constant weight was reached. The soil was analyzed for 12 parameters, namely total organic carbon, pH, total nitrogen, macro and micronutrients. Specifically, the following parameters were measured: Soil electrical conductivity (EC) and pH were determined in soil-water suspensions at a ratio of 1:5 and 1:2.5, respectively, using a conductivity meter and a pH meter (Czekała et al. 2016). Total nitrogen was determined using the Kjeldhal method (Czekała et al. 2016), while phosphorus was determined using the molybdovanadate-phosphate method. NO_3 and NH_4 content determination was done by mixing 1g of dry pulverized soil with 1ml of distilled water in a 2 ml Eppendorf tube. This mixture was shaken for 20 min and subsequently centrifuged for 5 min at 13,000 rpm. The samples were analyzed with a DR 3900 Spectrophotometer (Hach, Loveland, CO, USA) by using the manufacturer kits LCK 340 (assay range 5–35 mg/l, ISO 7890-1-2-1986) for NO_3 and LCK 303 (assay range 2–47 mg/l, ISO 7150-1) for NH_4. Organic matter content (OC) was determined by weight loss at 550°C for 8 h (Silva et al. 2014). Potassium, magnesium, calcium, and sodium were determined by flame atomic absorption spectroscopy (Peters et al. 2003). Total limestone is determined by the weight method against a strong acid. Attack of the limestone results in gas release of CO_2, the volume of which is measured (LANO: NF ISO 10693). Finally, the chloride content (Cl) in the soil was determined by the volumetric method described by Meldrum & Forbes (1928).

7.3.4 DNA extraction, Shotgun sequencing and functional annotation

DNA was extracted from three technical replicates using the CTAB protocol. In details, 5g of Soil was homogenized on a vortex mixer (S8A Stuart) at 2200 rpm for 5 minutes with 600µl of CTAB extraction buffer (2% cetyl trimethylammonium bromide, 1% polyvinyl pyrrolidone, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA). The homogenate was then transferred to a 60 °C bath for 30 minutes. Resulted lysate was centrifuged at 16000g for 5 minutes. The supernatant was mixed with 5µl of RNase A and incubated at 37 °C for 20 minutes. Thereafter,

the upper layer was transferred and mixed with an equal volume of chloroform-isoamylic alcohol (24:1 v/v) and centrifuged at 16000g for 5 minutes to separate the phases. The supernatant was then precipitated with 0.7 volume of ice-cold isopropanol, and incubated at -20 °C overnight. The DNA pellets obtained after centrifugation 14000g for 30 minutes at 4 °C were suspended in 50 µl of endonuclease-free water.

DNA libraries were sequenced on Illumina's NovaSeq platform, resulting in 2x150bp, paired-end reads, followed by shotgun metagenomics analysis. The resulting reads were quality- filtered using PRINSEQ 0.20.4 (Schmieder et al. 2011). Reads with bases that had a Phred score <15 were trimmed and those <75 bp were discarded. High-quality reads were imported into MetaPhlAn 3.0 (Beghini et al. 2021) to obtain quantitative taxonomic profiles at the species level. A subsystem-based approach was used to reveal the functional potential of the metagenome samples (Overbeek et al. 2005). Sequences were identified using the best BLASTx hit with a minimum alignment length of 50bp and an E $<1\times10^{-5}$ as the E-value cut-off from the annotation against the KEGG subsystem database. The functional annotations were filtered at subsystem level 1 and level 2 to create two functional profiles for each sample metagenome. The relative abundance of annotations within each subsystem was estimated for each metagenome sample by dividing the number of sequences classified as belonging to the subsystem by the total number of classified sequences per metagenome sample.

7.3.5 Extracellular self-DNA: extraction, amplification and sequencing

Extracellular DNA fractions were purified sequentially according to the modified protocol of Ascher et al. (2009). Specifically, exDNA was extracted by gently washing the soil with 5ml of 0.12M Na_2HPO_4 at pH 8 in 50ml Falcon tubes that were shaken horizontally at 80 rpm for 30 min. After 7500g centrifugation at 4 °C for 30 minutes, the supernatant was collected. The same procedure was repeated twice and both supernatants were pooled to a final volume of 15ml of unpurified exDNA. A purification step was then performed using a commercial extraction kit (DNeasy^R PowerMax^R Soil Kit, Qiagen, USA), following the manufacturer's instructions except for the step of incubating the sample in cell lysis buffer. At the end of purification, the DNA sample was suspended in 5ml of 10mM Tris solution.

Amplification of the purified exDNA was performed in a final volume of 25µl using 10µl of DNA extract, 1X concentrated OneTaq Hot Start Quick-Load, 2X Master Mix with standard buffer (New England Biolabs inc.) and 0.5µM of rbcL forward and reverse primers. These primers are capable of amplifying a 553bp fragment of the rbcL gene and are recommended by

the CBol Plant Working Group (2009) for plant metabarcoding. The primers selected were rbcLa_f 5'-ATGTCACCACAAACAGAGACTAAAGC-3' and rbcLa_rev 5'-GTAAAATCAAGTCCACCRCG-3' (Fahner et al. 2016). PCR conditions were 94 °C for 30s, followed by 40 cycles of 94 °C for 30s, 64 °C for 60s, 68 °C for 30s and a final step of 68 °C for 5min. A subsequent amplification run integrating relevant flow-cell binding domains and unique indices was performed using the NexteraXT Index Kit (Illumina). Amplification products were sequenced on the MiSeq instrument platform (Illumina, San Diego, CA) using 300bp paired-end and according to the manufacturer's instructions. Taxonomic classification was performed using a database of 181133 rbcL sequences downloaded from the NCBI Nucleotide Section on September 9, 2020.

The bioinformatics pipeline steps were as follows: Reads were trimmed using *cutadapt* (Martin, 2011) with default parameters to eliminate primer sequences. Low quality bases were removed from 3' with *erne-filter* (Del Fabbro et al. 2013) using default parameters and reads <60bp were excluded from further analysis. Reads with an error rate >1% were removed. Chimeric sequences were removed using the *uchime_denovo* algorithm (Edgar et al. 2011) implemented in *usearch*. Reads were clustered to a minimum identity of 97% using the *cluster_fast* algorithm implemented in *usearch* to produce representative sequences. BLAST was matched against the rbcL database without a minimum identity filter, with the lowest unambiguous taxonomic assignment among all possible blast hits. For hits with the same score indicating different lineages, the most frequent part was indicated. Sequences that could not be taxonomically assigned to *Streptophyta* were discarded.

7.3.6 Statistical analysis and data visualization

For the microbial data, alpha diversity metrics, i.e. number of reads, Margalef species richness index and Simpson index, were calculated and presented as boxplots using the software PRIMER 7 (Primer-E Ltd, Plymouth; UK) to assess variation in community composition at the lowest taxonomic levels. Heatmaps were used to represent the most abundant taxa in the fungal, bacterial and archaeal communities and were created using the ComplexHeatmap package implemented in R (version 3.3.2). Based on a resemblance matrix calculated using Bray-Curtis dissimilarity, non-metric multidimensional scaling (NMDS) analyses were performed based on the abundance of microbial communities using the metaMDS function of the Vegan package implemented in R. The significance of changes in composition between the two microbial communities was tested using PERMANOVA (999 permutations) with soil treatments as a fixed factor. The significance of variation in the alpha diversity metrics, soil biomass, and

exDNA abundance between treatments was assessed using the ANOVA test, and means were separated pairwise using the *post hoc* Tukey test to provide further detail on the level of significance between samples. We also applied a Spearman rank correlation test to compare *A. thaliana* biomass with soil chemical attributes, and a heatmap was generated using the ComplexHeatmap package in R. The level of significant differences was evaluated with $p < 0.05$. All statistical analyses were performed using STATISTICA 13.3 software.

To reveal the complexity of the microbiome and potential interrelationships among microbial community members, co-occurrence network analyses were performed for the microbial communities in the four different soils. To focus on the most abundant taxonomic species and reduce the influence of rare ones, only the 50 most abundant species were analyzed for each bacterial, fungal, and archaeal community. Pairwise correlations between taxonomic species were calculated using Spearman correlation with the Hmisc package in R. Based on statistical analysis, only strong and significant (Spearman's $r > 0.6$ or $r < -0.6$ and $p < 0.05$) correlations were considered. Network visualization was performed using Gephi software (version 0.9.2, Bastian et al. 2009). Each edge represents a robust and significant correlation and each node represents a taxonomic species. A set of integrative metrics was computed and compared to describe the network topology.

7.4 Results

7.4.1 *A. thaliana* performance and self-DNA content

The total biomass of *A. thaliana* was significantly affected by the different treatments on the conditioned soil (Fig.1A). The highest total biomass was recorded when the conditioned soil was sterilized by autoclaving with an average biomass of 11.6mg, followed by washing treatment with an average biomass of 5.7mg. However, the addition of activated carbon showed no significant differences compared to the control, and both had the lowest total biomass of 0.4mg and 1.5mg, respectively (Fig. 1B). In contrast, we note that the highest exDNA content was recorded in the soil with activated carbon at 0.44µg/g, followed by the control with a content of 0.18µg/g. Sterilized and washed soils, on the other hand, had the lowest content of exDNA at 0.04 and 0.09µg/g, respectively (Fig. 1B).

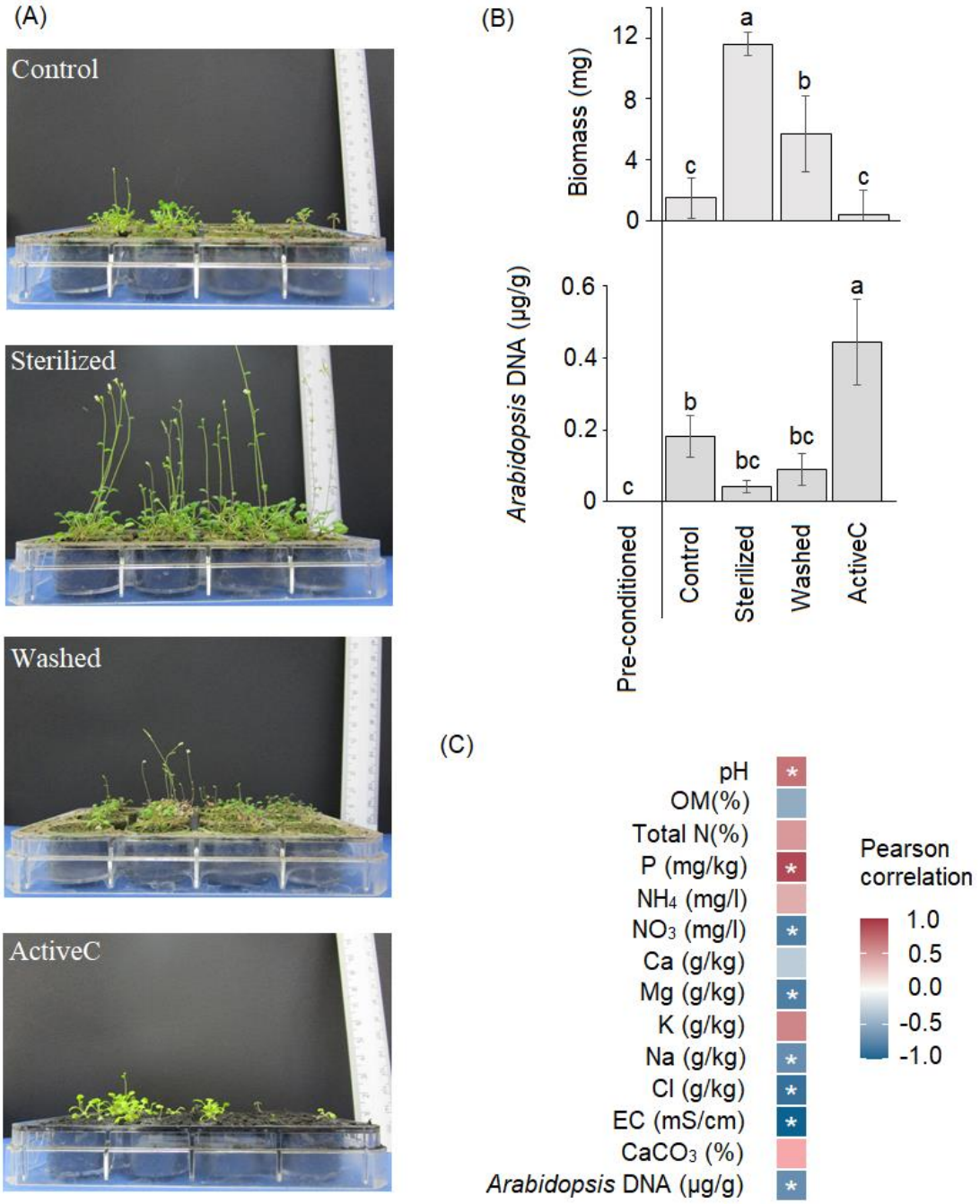

Fig 1. Shoots grown in different soil treatments at the end of the experiment (A). Total biomass (mg/pot) of *Arabidopsis thaliana* and self-DNA in each treatment at the end of the experiment (B). Heatmap of correlation matrix based on the Pearson correlation between soil chemical properties with the total biomass (C).

7.4.2 Soil chemical properties and correlation with biomass

The soil chemical parameters showed significant differences between the treated soils (Table S1). Specifically, compared to the other soils, the sterilized soil had the lowest electrical conductivity and NO_3 content, while the P content was the highest. On the other hand, the soil

with activated carbon had the highest organic matter and NO_3 contents, while NH_4 content was the lowest. Moreover, total limestone was significantly higher in the control and washed soils, while chloride content was significantly higher in the control and activated carbon soils compared to the other soils. However, no significant differences were observed between the soils in terms of pH, Mg, K, total N and Na. Furthermore, Spearman correlation with soil chemical parameters (Fig. 1C) showed a strong significant positive correlation between total biomass of *A. thaliana* and pH as well as P content, while a strong negative correlation was observed with NO_3, Mg, Na, Cl, EC, and self-DNA content in soil.

7.4.3 Metagenomics: diversity, abundance and composition

Our results showed that the number of reads was significantly highest in sterilized soil followed by activated carbon soil, while it was significantly low in control and washed soil (Fig. 2). On the other hand, the species richness index was significantly higher in washed soil than in sterilized soil. Moreover, Simpson's index was significantly higher in sterilized soil than in control.

The results of our shotgun sequencing showed that in all soils, Bacteria were the dominant kingdom at 98%, followed by Archaea (1.5%), Fungi (1%), and Eukaryota (0.5%) (Fig. 3A). At the phylum level (Fig. 3B), the bacterial community varied among the treated soils (Fig. 3B). In details, all the soils contained mainly *Proteobacteria*, from 63.29% in the sterilized soil, followed by 55.78% in the washed soil, 50.21% in the activated carbon soil and 49.4% in the control. On the other hand, the highest abundance of *Planctomycetes* was found in sterilized soil at 9.11% while the lowest abundance was recorded in activated carbon soil at 2.94%. *Firmicutes*, on the other hand, were most abundant in sterilized soil at 5.1% compared to washed soil at 2.51%. Moreover, *Bacteroidetes* were more abundant in sterilized soil (3.2%) than in activated carbon soil (1.23%), while *Actinobacteria* were very abundant in activated carbon soil (39.01%) and less abundant in sterilized soil (17%). At the level of archaea, the phylum *Euryarchaeota* dominated in all the studied soils with a frequency of 91.27%, 84.6%, 82.6% and 76.72% in sterilized, activated carbon, washed and control soils, respectively. Moreover, *Thaumarchaeota* were highly abundant in control at 20.5% while they were very low in sterilized soil at 4.05%. *Crenarchaeota*, on the other hand, were very abundant in the sterilized soil at 4.32% compared to the other soils. Furthermore, the fungal community in the treated soils showed significant variation at phylum level. In particular, all the soils were dominated by *Ascomycota* with abundances of 93.01%, 91.96, 89.59% and 88.38% in the control, washed, sterilized and activated carbon soils, respectively. *Basidiomycota*, however,

were very abundant in both activated carbon and sterilized soils with 11.3% and 9.94%, respectively, while they were low in control and washed soils with 6.6% and 7.73%, respectively.

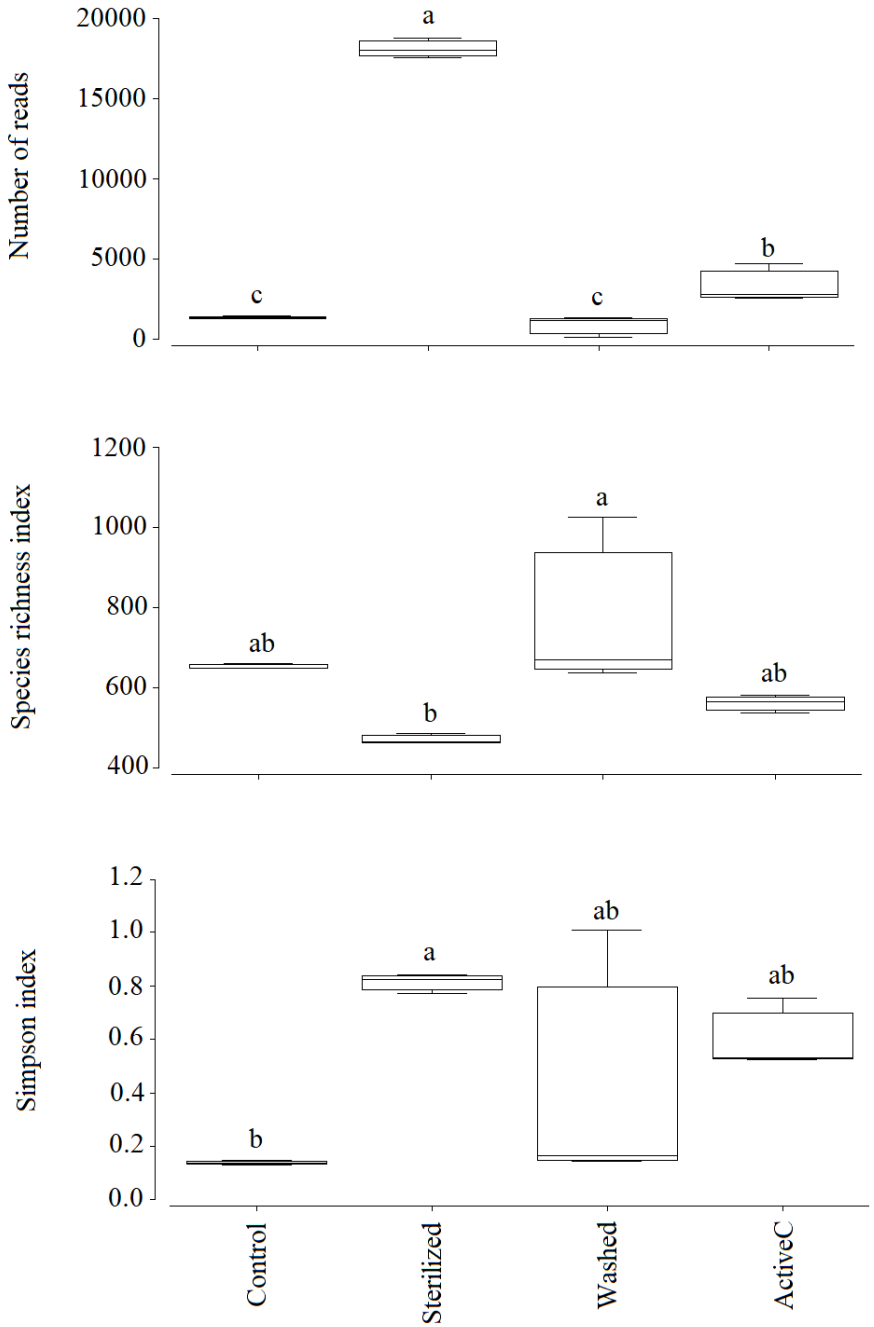

Fig 2. Box plots showing the variation in the numbers of reads, species richness, and Simpson indices for the overall microbial communities for each treatment. Different letters indicate significant ($P < 0.05$) differences in the indices. The lower and upper bounds of the boxplots show the first and third quartiles (the 25th and 75th percentiles); the middle line shows the median, whiskers above and below the boxplot indicate inter-quartile range.

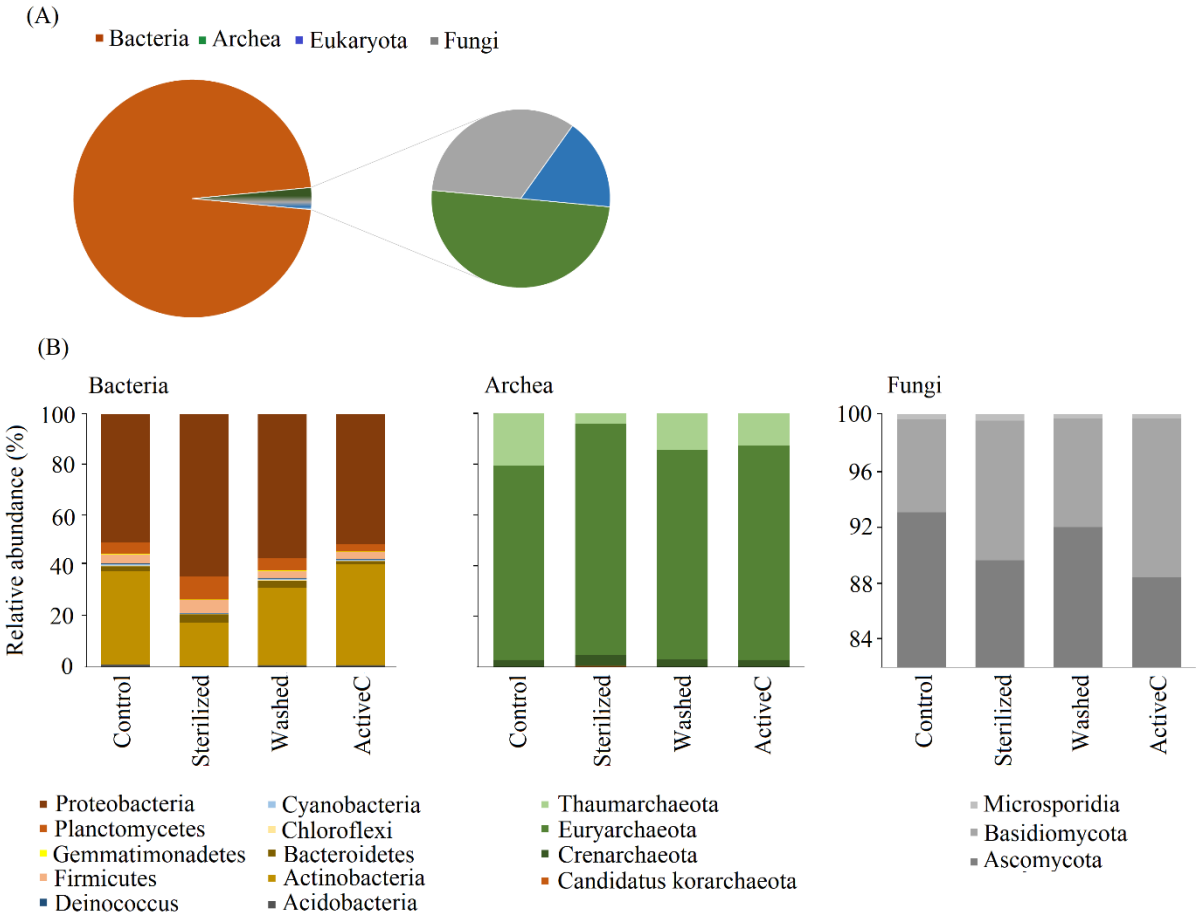

Fig 3. The relative abundance of various bacterial, fungal and archaeal phyla for each treatment.

At the lowest taxonomic level, the bacterial heatmap of the relative abundance of the most frequent species showed a large difference between soils, especially compared to the sterilized soil (Fig. 4). In particular, the sterilized soil showed a high divergence compared to the other soils due to the dominance of a group of bacterial species that mainly included *Sphingopyxis fribergensis*, *Devosia*, *Georhizobium profundi*, *Pseudomonas stutzeri*, *Brevundimonas* and *Microbacterium*. The washed soil, on the other hand, was characterized by high presence of *Streptomyces fradiae*, *Pseudomonas stutzeri*, *Microvirga ossetica*, *Lysobacter* and *Sorangium cellulosum*. In contrast, the soil with activated carbon contained mainly a group of bacteria including *Streptomyces fradiae*, *Sinorhizobium fredii*, *Streptomyces scabiei*, *Sorangium cellulosum* and *Rhodopseudomonas palustris*. However, the control soil had the highest abundance of *Streptomyces fradiae*, *Sorangium cellulosum*, *Conexibacter woesei*, *Luteitalea pratensis* and *Microvirga ossetica*. The heatmap of archaea, on the other hand, showed a significant variation between sterilized soil and other soils, especially that most of the common archaeal species were less abundant in sterilized soils. Nevertheless, the fungal heatmap showed a clear difference between the soils, especially compared to the sterilized soil.

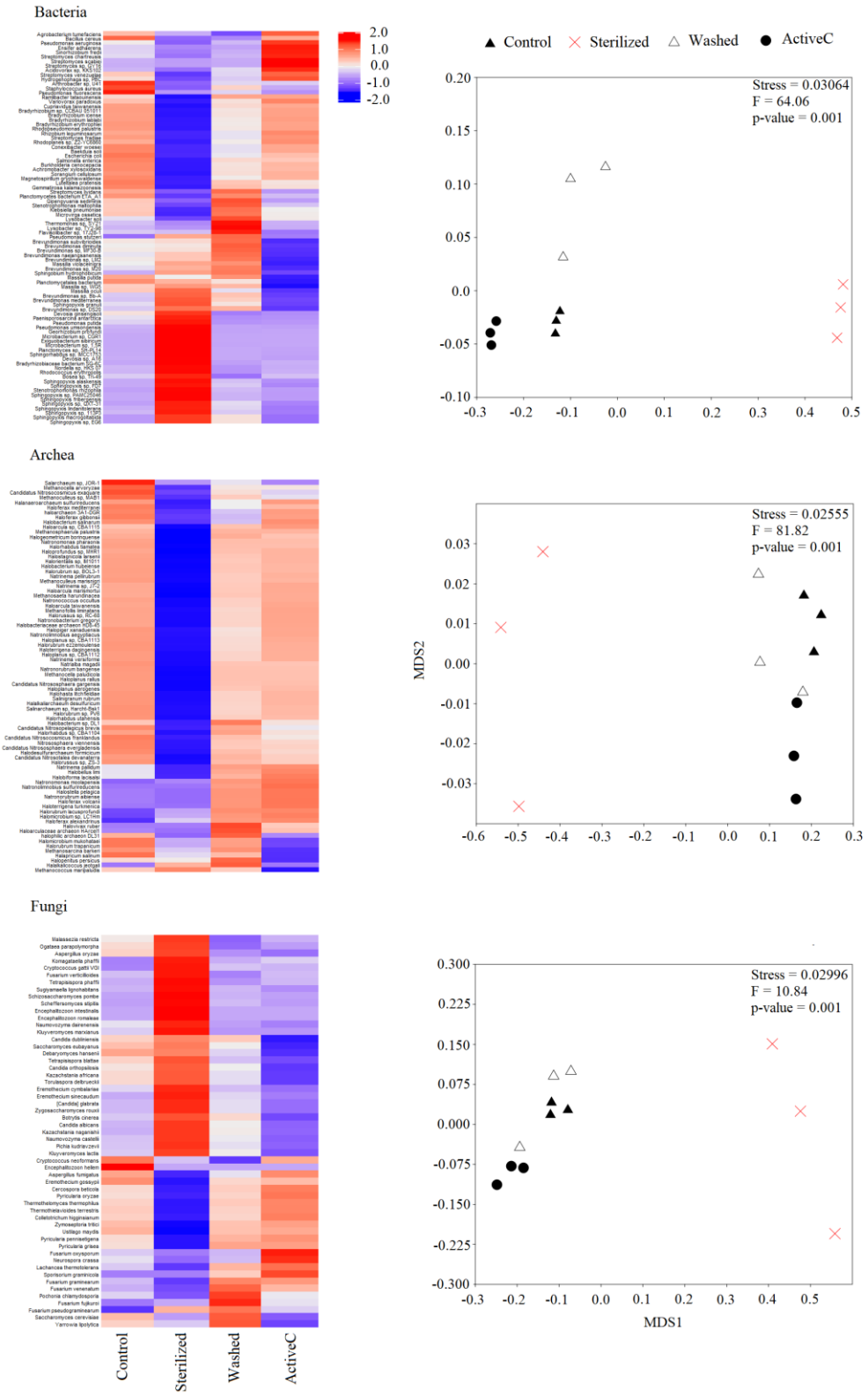

Fig 4. Heatmaps showing relative abundance of the most frequent species in the bacteria, fungal, and archaeal communities for each treatment along with nonmetric multidimensional scaling (NMDS) plots of bacteria, fungi and archaea communities for each treatment. MDS axis 1 and MDS axis 2 represent the two axes of the two-dimensional ordination space. Each point represents the microbiome of one replicate of the treatment. The stress-level shown in each plot indicates how well the individual

distances between objects are represented (between 0 and 1; the closer to 0, the better are original data points represented in the ordination space).

Specifically, the sterilized soil was characterized by a group of fungi including *Colletotrichum higginsianum*, *Aspergillus oryzae*, *Eremothecium gossypii*, *Pyricularia oryzae*, and *Botrytis cinerea*. However, the washed soil was characterized by high presence of *Pyricularia pennisetigena*, *Ustilago maydis*, *Sporisorium graminicola*, *Pochonia chlamydosporia* and *Pyricularia grisea*. On the other hand, the control soil had high abundance of *Pyricularia pennisetigena*, *Pyricularia grisea*, *Zymoseptoria tritici*, *Fusarium venenatum* and *Fusarium oxysporum*. However, the soil with activated carbon contained a group of fungi mainly *Cercospora beticola*, *Colletotrichum higginsianum*, *Fusarium verticillioides*, *Zymoseptoria tritici* and *Pyricularia grisea*. Furthermore, non-metric multidimensional scaling (nMDS) ordinations based on the Bray-Curtis similarity matrices showed a clear separation of bacterial, archaeal and fungal community structure between the sterilized soil and other soils ($p < 0.05$, Fig. 4).

7.4.4 Co-occurrence network

We constructed four co-occurrence networks for the four soil treatments (Fig. 5) and calculated seven topological parameters to assess interactions among microbial species in each of the four networks (Table S2). The microbial networks contained 184 nodes and 2429 edges in the control soil, 188 nodes and 3324 edges in the sterilized soil, 183 nodes and 3826 edges in the washed soil, and 178 nodes with 2165 edges in the soil with activated carbon. The network of the sterilized soil had the highest positive correlations of 72.56%, while the network of the washed soil had the lowest positive correlations of 50.89%. Moreover, the heterogeneity of the network was higher in the control and sterilized soils than in the others, while the centralization of the network was lowest in the soil treated with activated carbon. On the other hand, the network diameter was higher in the control and activated carbon soils than in the others. Moreover, the characteristic path length was highest in the control and lowest in the washed soil, while the clustering coefficient and network density showed no differences among the treated soils. Nevertheless, the modularity was highest in the washed soil followed by the activated carbon soil compared to the other soils.

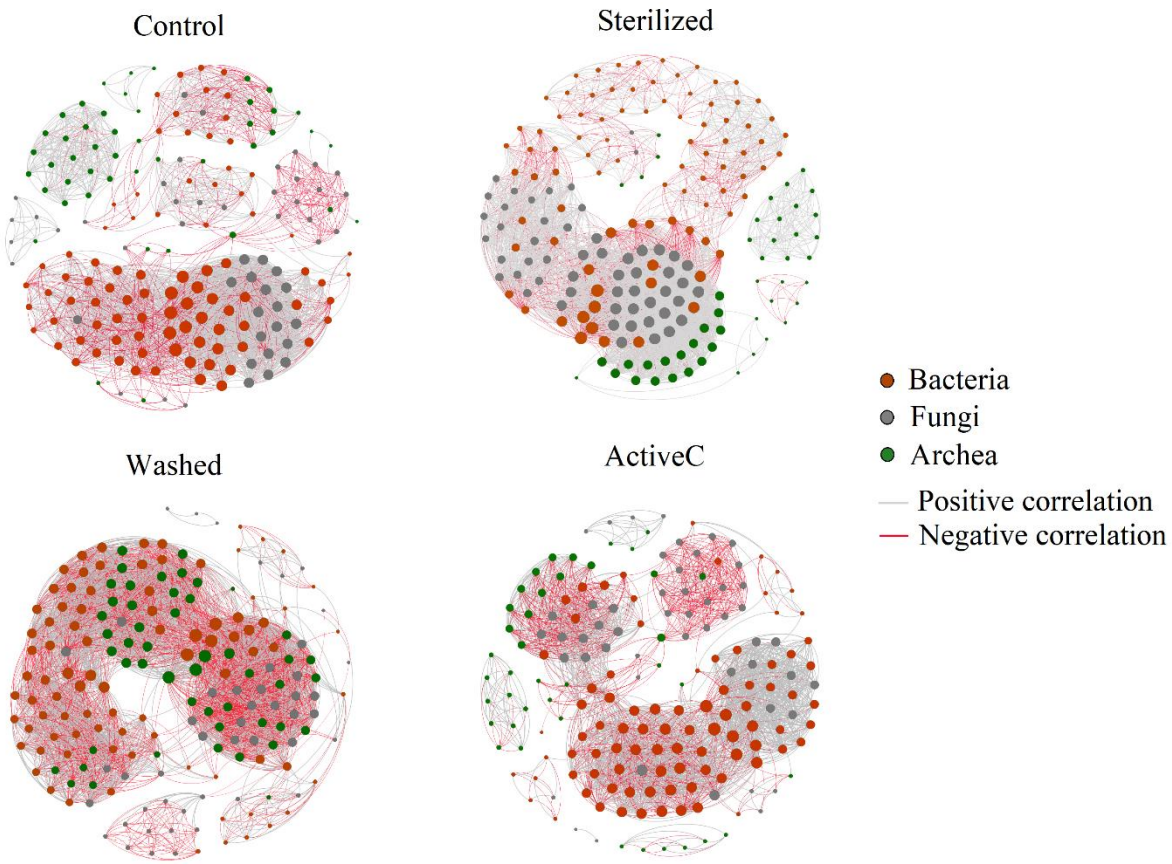

Fig 5. Correlation network analysis showing potential interactions between bacterial, fungal and archaeal communities. The lines connecting nodes (edges) represent co-occurrence relationships. The length of the edges represent the strength of correlation. Nodes correspond to the microbial species coloured differently by kingdom level. The connection stands for significant (P-value<0.05) correlations. The size of each node is proportional to the ASV relative abundance, only the top 50 OTUs were kept for each kingdom.

7.4.5 Metagenomics: soil functional diversity

Heatmap presenting the most abundant functional genes belonging to KEGG subsystem 2 shows that the sterilized soil has a significant variation compared to other soils (Fig. 6A). In details, it shows that sterilized soil contains a higher number of genes belonging to different groups of subsystem 1, especially genes belonging to membrane transport, stress response and defence, cellular processes, metabolism, and regulation and cell signalling. Moreover, the non-metric multidimensional scaling (nMDS) ordinations based on the Bray-Curtis similarity matrices showed a clear separation between the ordination of the sterilized functional genes and the other treated soils (Fig. 6B).

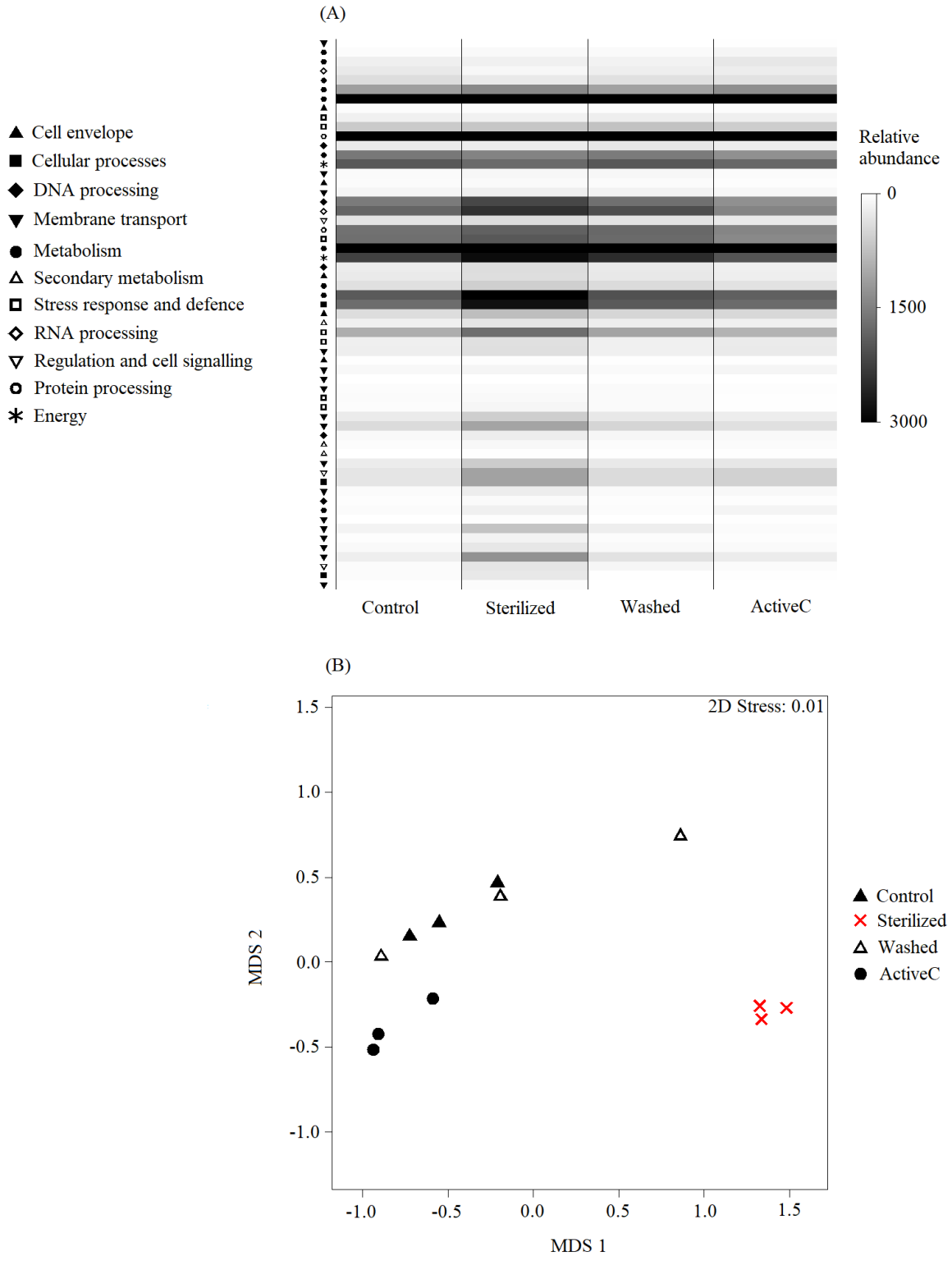

Fig 6. (A): Heatmap showing relative abundance of genes sub-categories based on Kegg path in the different soil treatments. (B): Nonmetric multidimensional scaling (NMDS) plots of overall identified genes for each treatment.

7.5 Discussion

In this study, we compared the performance of *A. thaliana* on soil previously conditioned by the same plant and then subjected to different treatments, namely sterilization, washing, and addition of activated carbon. Our results show that the soil legacy left by the conditioning phase was significantly affected by the treatments exhibited, as the performance of the plants differed significantly between these treatments. We found that the highest biomass was achieved when the conditioned soil was sterilized, followed by the washing treatment, while the addition of activated carbon was the lowest and showed no significant difference compared to the control without treatment. Our results showed that *A. thaliana* suffers from a strong negative plant-soil feedback and that sterilization and washing of the soil helped alleviating it. In agreement with our results, Bukowski et al. (2018) have concluded also, in a different scale experiment, that *A. thaliana* suffers from strong negative feedback at all taxonomic levels. In addition, soil sterilization has been known, since the 1960s, to reduce or prevent the increase in negative plant-soil feedback (Hoestra, 1968; Savory, 1966). However, soil-washing treatment has never been tested within the feedback effect context. It is worth noting that the negative PSF occurs mainly in terrestrial systems, while it was rarely observed in aquatic environments (Mazzoleni et al., 2007), which in a way supports our results, since washing under running water mimics the aquatic environment. On the other hand, activated carbon has shown the potential ability to suppress plant diseases (Elmer & Pignatello, 2011) and increase plant productivity (Spokas et al., 2012; Kolton et al., 2016). However, our results show that its addition did not contribute to plant recovery. In contrast to our results, Wang et al. (2020) showed that the application of activated carbon have succeeded to alleviate the negative PSF in a Sanqi (*Panax notoginseng*) production system.

Plant-soil feedback is a complex, multifactorial phenomenon. To explain the observed behavior of the plants in our experiment, we explored the three main hypotheses proposed in the literature to lead towards negative PSF, namely nutrient depletion (Howeler, 1991), shift in soil microbiota associated with the accumulation of pathogens in the soil (Klironomos, 2002; Manici et al. 2013) and the release of autotoxic compounds, i.e., self-DNA, during decomposition of plant litter (Mazzoleni et al., 2015). In the response phase of this experiment, we used the entire conditioned soil to exhibit the response phase instead of using 10% of the conditioned soil as inoculum, as is the case in most feedback studies. The advantage of this method is that it allows exploring the chemical legacies produced by the decomposition of litter and root exudates, rather than being limited to the microbial legacies alone. Indeed, several previous studies have found that soil chemistry helps explain much of the variation in the

strength of PSF effects (Ehrenfeld et al. 2005). However, our results indicate that the direction and strength of the feedback between treatments is not due to soil chemical properties, as we found no significant differences between soils in pH, Mg, K, total N, and Na. In addition, we found that the soil with activated carbon, which suffered from a strong negative feedback effect, had the highest organic carbon content and was not different in terms of P content from the washed soil, which had a strong positive feedback effect. Thus, supporting the fact that priority effects in plant communities are not just a matter of resource availability. In agreement with our results, Harrison & Bardgett (2010) showed that the effects of PSF occurred independently of soil physicochemical conditions in mixed grassland communities.

On the other hand, the abundance of functionally important microbial phyla was affected by the treatments, especially sterilization. Soil sterilization alters biotic and abiotic soil properties and provides nutrient fluxes resulting from rapid mineralization of dead microbes (Troelstra et al. 2001). In addition, organic phytotoxic compounds can be thermally degraded. Therefore, the greatest availability of nutrients or degradation of toxic compounds induced by soil sterilization can simultaneously reduce the negative effects of PSF and promote growth (Troelstra et al. 2001). Our results showed that the sterilized soil contained the highest amount of P. Previous studies have also shown that changes in soil P concentration are the main factor leading to changes in microbial community composition (Wei et al. 2020). We found that sterilized soil contained greater amounts of *Proteobacteria*, *Planctomycetes*, *Firmicutes*, *Bacteroidetes*, *Euryarchaeota*, *Crenarchaeota*, and *Basidiomycota*, while *Actinobacteria* and *Ascomycota* were the least represented. Bacteria belonging to *Proteobacteria* and *Firmicutes* may use nutrient sources to improve soil quality (Han et al. 2020), as *Proteobacteria* are generally considered more copiotrophic, while *Actinobacteria* are considered oligotrophic in soil. Moreover, these results could explain the difference in observed growth between sterilized and washed soil, as the latter contained fewer *Proteobacteria* and the fewest *Firmicutes* compared to sterilized soil. Similar to our results, several previous studies have shown that soil washing drastically affects the microbiome that develops in a new environment (Howard et al. 2017). In addition, *Euryarchaeota* were found to be able to oxidize methane (Michaelis et al., 2002), fix nitrogen (Raymond et al., 2004), reduce nitrate (Cabello et al., 2004), and metabolize sulfur and iron (Edwards et al., 2000), and their beneficial effects could also explain the increased growth in the sterilized soil. Our results show that the activated carbon soil had similar bacterial and archaeal content to the control soil, as both had higher percentages of *Actinobacteria* and *Euryarchaeota*. Similarly, previous studies have proved that the level of

Actinobacteria in the soil is positively correlated with activated carbon amendment (Jaiswal et al. 2017; Wang et al. 2020).

At the lowest taxonomic level, our results show that all soils harboured a significant amount of fungal pathogens, even in the sterilized soil where they were allowed to accumulate during the response phase. In general, evidence that soil-borne pathogens are consistently isolated from symptomatic plants supports the pathogenic hypothesis causing the negative PSF. However, our results showed that pathogens were found in soils where growth was extremely boosted, i.e., sterilized and washed soils. Recent studies have shown that the net effect of plant-soil feedback is the balance between beneficial and harmful microbes. Notably, the type of mycorrhizal association with plant species explained much of the variation in negative PSF, with arbuscular mycorrhizal trees suffering a stronger negative PSF than ectomycorrhizal trees (Bennett et al. 2017). However, our model plant does not generally form symbiotic relationships with mycorrhizal fungi. Yet, we found many beneficial bacteria and archaea such as *Georhizobium profundi*, the denitrifying *Pseudomonas stutzeri*, *Streptomyces fradiae*, and *Microvirga ossetica* in our soils. This may suggest that the net outcome for *A. thaliana* growth depends on antagonistic and synergistic interactions within the soil microbiome. The changes in the relative abundance of microbial species belonging to these functional groups can strongly influence plant growth or health (van der Putten et al. 2016; Hannula et al. 2017), since in our case bacteria and archaea were very dominant in the soil compared to fungi. The presence of soil-borne fungal pathogens in the sterilized and washed soils where growth was largely high, and the ability of these treatments to eliminate exDNA in the soil by either degradation or leaching, dwarfs the microbial origin of the observed negative PSF and sheds light on the autotoxicity theory led by extracellular self-DNA.

The recent observations by Mazzoleni et al. (2015) on inhibitory effects by extracellular self-DNA in plants opened new perspectives for understanding the autotoxicity of litter and negative PSFs. The authors reported significant evidence that fragmented exDNA has a concentration-dependent and species-specific inhibitory effect on plant growth. Our results suggest that *A. thaliana* exDNA accumulated in the soil after the conditioning phase, whereas it was undetectable before this phase. Such accumulation of DNA molecules is mainly due to the degradation of organic material, which occurs through excretion from living cells (Nielsen et al. 2007). Moreover, our results show that the correlation analysis between plant biomass and self-DNA was significantly negative, confirming the occurrence of the previously reported species-specific inhibitory effect. Interestingly, we observed a concentration dependence of

inhibition by extracellular self-DNA, as our results show that sterilized and washed soils had the highest growth and lowest concentration of self-DNA, while control and activated carbon soils had the lowest growth and highest concentration of self-DNA, with activated carbon soil in particular having the highest level of self-DNA. Previous studies have shown that autoclaving affects DNA by decreasing the total content of DNA and causing its fragmentation, degradation, and denaturation (Maity et al. 2009; López-Andreo et al. 2012). In addition, a previous study has shown that environmental DNA concentration decreases by 16% per hour under tap water (Maruyama et al. 2014). On the other hand, activated carbon has been reported to have a high affinity to adsorb environmental DNA, which increases with pyrolysis temperature (Wang et al. 2014; Fang et al. 2021). These studies could explain the extracellular self-DNA concentration found in our soils, as activated carbon soil had the highest concentration and both sterilized and washed soils had the lowest. Moreover, our study provided evidence for the hypothesis that extracellular self-DNA has an inhibitory effect on conspecifics. Indeed, previous studies have demonstrated an association between negative PSF and the occurrence of species-specific pathogenic microbial communities (Packer & Clay, 2000; Kardol et al. 2007), so it should be seriously considered that weakening a plant as a result of exposure to extracellular self-DNA may ultimately increase its susceptibility to pathogen attack (Mazzoleni et al. 2015). Such negative effects of self-DNA have been attributed to several putative mechanisms (Veresoglou et al. 2015), including signalling and self-recognition (Duran-Flores & Heil, 2015; Bhat & Ryu, 2016), plant root defence (Hawes et al. 2011), and molecular patterns associated with microbes or damage (Panstruga, 2016; Vega-Muñoz et al. 2018; Heil & Vega-Muñoz, 2019). A recent study by Chiusano et al. (2021) showed that exposure of *A. thaliana* to its self-DNA limits cell permeability, which impairs chloroplast function and reactive oxygen species production, eventually leading to cell cycle arrest. Furthermore, Bonanomi et al. (2022, under review) presented for the first time under field conditions a novel method demonstrating that self-DNA, but not heterologous one, exerts acute toxic effects on *Alnus glutinosa* L. roots in a closed system. Investigating the molecular mechanisms behind the observed inhibitory phenomenon is indeed a major challenge that we had to face. In this study, we succeeded for the first time in quantifying extracellular self-DNA directly from soil and attributing the observed negative PSF to the concentration of this exDNA in soil by applying such treatments that affect its presence and consistency. The previous evidence that self-DNA is toxic, as well as our results reported here linking the observed negative PSF to the concentration of self-DNA, and its known ability to limit conspecific plant

root cells (Chiusano et al. 2021), suggest that inhibitory effects of self-DNA should be considered as a further mechanism to explain the species-specific negative PSF.

7.6 Conclusion

Here we have provided evidence for the chemical basis of autotoxicity that produces negative PSF. Since the applied treatments are known to affect DNA persistence in soil, our results showed a concentration dependence of inhibition by extracellular self-DNA, as we observed that sterilized and washed soils had the highest growth and lowest concentration of self-DNA, while control and activated carbon soils had the lowest growth and highest concentration of self-DNA, thus confirming that extracellular DNA has species-specific inhibitory effects on plants. Sterilization treatment, two sides of the same coin, can affect both DNA and soilborne pathogens in the soil, explaining the increased growth compared to soil washing, which only affects DNA from both. Therefore, our results makes it decent to suggest that weakening a plant by exposing it to extracellular self-DNA may ultimately increase its susceptibility to pathogen attack, which was the case in the control and when activated carbon was added.

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