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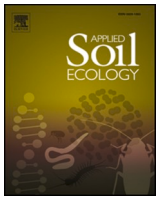

Implications of nurse species in mixed forest plantations management on soil fungal community diversity

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ABSTRACT

Mixed plantations provide numerous benefits in terms of ecosystem and socioeconomic services, as well as on soil chemical and biological parameters; thus, a forest management allowing to recover overexploited soils is highly recommended. Although nurse species may deeply affect soil properties, few studies are present in literature assessing their role. A study site characterized by a mixed plantation established on a former agricultural area was selected in order to evaluate the impact of a specific forest management on marginal soils, with a special emphasis on the role of nurse species. The intercropping systems investigated feature two economically important species, *Populus alba* and *Juglans regia*, along with one of following nurse trees, *Alnus cordata*, *Elaeagnus umbellata* (both N-fixing species), and *Corylus avellana*. Each stand was replicated three times, and an adjacent agricultural field was included for comparison. Methodologically, topsoils (0–10 cm of depth) were sampled and several chemical and biological parameters determined. Fungal taxa, as well as fungal ecological guilds and their functional roles, were identified by means of metabarcoding analysis. Ectomycorrhizal fungi dominated tree consociations (53.5 %), while non-mycorrhizal saprophytes dominated the arable, control soil (5.3 %). Two-Block Partial Least Squares showed differences both among tree consociations, where the presence of the *Alnus cordata* resulted in the highest concentration of organic carbon (19.10 ± 1.8 mg g^{-1}), total nitrogen (1.78 ± 0.1 mg g^{-1}), lignin (11.25 ± 1.1 mg g^{-1}), cellulose (1.54 ± 0.2 mg g^{-1}), and bioavailable phosphorus (8.99 ± 1.2 mg kg^{-1}), as well as fluorescein diacetate hydrolase enzyme activity, and between tree consociation and the arable land. Thus, the utilization of *Alnus cordata* as a nurse species seems to be the best solution for a forest management capable of improving soil chemical and biological quality, providing a viable strategy for the restoration of marginal soils, particularly in a climate change scenario.

1. Introduction

Given the extensive range of ecosystem services provided by soils, whose loss threatens ecosystem health and, in turn, human well-being, protecting and recovering overexploited soils is a very current concern in light of the ongoing climate change scenario.

Agriculture may pose a real threat to soil health, especially when carried out in a not sustainable way. Soil organic matter (SOM) loss, erosion, compaction, salinization, and pollution have a significant negative influence on biodiversity and, in turn, on soil functionality (Conti et al., 2016; de Graaff et al., 2019), at both European (European Commission: Joint Research Centre, 2024) and global (FAO and ITPS,

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2015) scale. For this reason, the adoption of sustainable management practices aimed at preserving, or even improving, the SOM content is fundamental to mitigate soil degradation and biodiversity loss, as well as to restore soil fertility (Lal, 2004; Minasny et al., 2017; Plaza et al., 2018; Smith et al., 2020), especially in low-SOM soils characterizing the Mediterranean areas where climate change will exacerbate agronomic and pedological drought phenomena (Sofia et al., 2024, 2025).

Recent research has revealed that belowground communities are remarkably diverse; estimates suggest that 1 g of soil can harbour tens of thousands of bacterial taxa, up to 200 m of fungal hyphae representing thousands of fungal taxa, and a wide range of micro-, meso-, and macrofauna (Bardgett and Van Der Putten, 2014; Roesch et al., 2007). Such a biological diversity plays a critical role in the management of terrestrial ecosystems and has an impact on a number of ecosystem processes, including (i) the cycling of energy and nutrients that support plants and animal growth, (ii) the maintenance of water balances that affects aquatic organisms and ecosystem health (Cavicchioli et al., 2019), and (iii) the dynamics of eco-evolution in plant and underground communities in response to climate change (Bardgett and Van Der Putten, 2014). Due to their emphasis on trophic strategies, ecological guilds, also known as "functional groups", offer a different view on community composition than measures based on species richness or taxonomic identification (Nguyen et al., 2016a). Additionally, even when there is no direct overlap in species composition, the use of guilds enables comparisons among various ecosystems.

Soil microbial communities play a key role as chemical engineers, and they can act as biological regulators (Saccá et al., 2017). In particular, fungi can be classified into three groups: (1) biological controllers, which regulate diseases and pests, and enhance the growth of other organisms (e.g., mycorrhizal fungi) (Ramachandra et al., 2018); (2) ecosystem regulators, responsible for soil structure formation and modification by regulating the soil physiological processes; (3) decomposers and compound transformers (Gardi and Jeffery, 2009; Swift, 2005). Because their diversity and activity are influenced by a number of abiotic (e.g., soil pH, moisture, salinity, structure, and temperature) (Li et al., 2020) and biotic (e.g., plants and other organisms) (Kang et al., 2018) factors, as well as by land use (Li et al., 2018; Moghimian et al., 2017), fungal communities are particularly sensitive to both natural and human-induced environmental changes. The UN 2030 Agenda for Sustainable Development and the UN Convention on Biodiversity's Strategic Plan for 2020 (Bach et al., 2020), two initiatives that identify soil biodiversity as the core of natural solutions for climate and humanity well-being, have the preservation of natural areas, the restoration of degraded ecosystems, the use of sustainable agricultural practices, and the adaptation of urban areas for nature and people within their goals. From an international perspective, as the Paris Agreement signatories revise their Nationally Determined Contributions, nature-based solutions (NbS) are identified as key to meet global goals for climate and biodiversity (Seddon et al., 2020). The reforestation of over-exploited and abandoned agricultural fields is listed among the NbS given the numerous benefits that induces to the soil, such as erosion and floods reduction, water retention, and increase in SOM and biodiversity (Seddon et al., 2020).

Plant-fungal interactions are crucial for ecosystem functioning, but the impact of plant diversity on microbial communities in forest ecosystems remains unclear. A key challenge is understanding the bidirectional relationship between plant and fungal diversity (Allen et al., 1995). Plant species richness positively influences soil microbial richness, including ectomycorrhizal (EM) fungi, due to increased environmental heterogeneity (Dickie, 2007). This is supported by studies showing a positive correlation between plant and fungal diversity at the local scale (Tedersoo et al., 2016). Among environmental factors, tree host specificity has been recognized to be an important driver of symbiotic (Buée et al., 2009) as well as saprotrophic fungi (Lang et al., 2011). The chemical composition of plant litter and root exudates, which changes among plant species, models the rhizosphere microenvironment and affects microbial community composition (Zhalnina et al., 2018), including fungal diversity. Previous studies (e.g., Öpik et al., 2009) suggested that partner specificity in arbuscular mycorrhizal symbioses may occur at the level of ecological groups, rather than at the species level, for both plant and fungal partners. However, other studies (e.g., Nguyen et al., 2016b) have found no significant effect of plant species richness on fungal richness at the local scale. This discrepancy highlights the complexity of plant-fungal interactions and the need for further research to fully understand these relationships.

In terms of regeneration efforts or arboriculture plantings in general, monocultures were the most common forest structure used for a long time. More recently, smallholders and landowners are paying more attention to mixed-species plantations as they can be more productive and offer more advantages in terms of biodiversity and economy respect to monocultures (Liu et al., 2018). As a result, finding a forest management system able to improve soil chemical and biological parameters, as well as to provide owners with an economic yield made up of wood, fruit or biomass, could prevent land abandonment and represent a successful approach that complies with circular economy standards in a climate change scenario. Many mixed-plantations involved nurse species since their effect is based on the key role of positive plant-plant interactions, such as generating microsites for species recruitment if facilitative effects on seedling survival or growth outweigh the competitive ones (Fedriani et al., 2019). The intercropping with nurse plants may offer protection to seedlings by buffering microclimatic conditions, e.g., by ameliorating extreme temperatures, reducing direct solar radiation or retaining soil moisture (Tapella et al., 2021).

Although previous works found that mixed-species plantations improve soil properties compared to monoculture plantations (Guo et al., 2023; Liu et al., 2018), sometime addressing the effects of accessory species on valuable ones (e.g., Venier et al., 2023), literature still lacks studies assessing the impact of nurse species on soil chemical and biological features, including fungal community composition. In this work, we focused on a site characterized by a mixed plantation set up on a former agricultural field. Four tree associations and one agricultural field, featuring the presence of nurse species like *Alnus cordata* and *Elaeagnus umbellata* (nitrogen-fixing species) as well as *Corylus avellana*, intercropped with valuable species, such as *Populus alba* and *Juglans regia*, are investigated. Valuable species were identified due to their importance in wood production in Italy, while accessory species were selected on the basis of their contrasting effect on valuable species. The relationship between soil quality and tree species productivity in both forest stands and an adjacent farmland has been already investigated in previous works (Danise et al., 2021a; Niccoli et al., 2021). These studies showed that *Elaeagnus umbellata* improved the growth of both target species, whereas the Italian alder (*Alnus cordata*) was associated with a higher soil quality. Lignin quality and quantity (Danise et al., 2020), as well as the intercropping impact on leaf metabolic profiles (Danise et al., 2021b), were also assessed. Here, the fungal community role in the different tree associations is evaluated, investigating the relationship among soil fungal community, soil chemical characteristics and total soil microbial activity. As plant-plant relationship is mediated by soil (Pugnaire et al., 2004), we test the hypothesis that different accessory species have a different impact on soil features including microbial communities. A multivariate approach that examines the covariance between variables associated with soil chemical and biological properties (e.g., organic carbon, total nitrogen, available phosphorous, lignin and cellulose content, microbial activity assessed by an enzymatic assay) and fungal communities (e.g., fungal guilds). Furthermore, we hypothesise that N-fixing nurse species will have a different impact on soil chemical and biological properties, including fungal guild composition, compared to other nurse species.

2. Material and methods

2.1. Study site and soil sampling

The experimental site is located in Empoli (Tuscany, Central Italy) (43°40′29″ N, 10°55′21″ E), at 35 m a.s.l. The mean annual precipitation is 850 mm, while the mean annual temperature is 15 °C. The soils developed on recent (Holocene) fluvial deposits and are Fluventic Haplustepts, coarse-loamy, mixed, thermic (U.S. Soil Taxonomy), according to the 1:250,000 soil map by the Regione Toscana. The study site was a mixed tree plantation (7.44 ha) established in 1996 on formerly cultivated land. The tree plantation comprises different and neighbouring stands/mixed intercropping where poplar (*Populus alba* L., Salicaceae) and walnut (*Juglans regia* L., Juglandaceae), widely used in Italy for timber production, were planted together using a triangular layout with a distance of 8 m (179 trees per ha), and intercropped with different nurse trees and shrubs, using a rectangular layout of 3.5–4 m (715 trees per ha) and maintaining the same layout and density of the two main tree species. The individuals of the same species were all the same genotypes.

In particular, the following planting plots (stands) were compared using a randomized blocks design with three replications:

- poplar and walnut (PJ stands) were planted using an intimate mixture;
- poplar, walnut intercropped with Italian alder (*Alnus cordata* (Loisel.) Duby, Betulaceae) (PJA stands), known as a nitrogen(N)-fixing tree species given the association with symbionts capable of fixing N, endemic to the forests of southern Italy, and widely used in tree farming plantations;
- poplar, walnut intercropped with hazel (*Corylus avellana* L., Betulaceae) (PJC stands), a native shrub common in the surrounding forests;
- poplar, walnut intercropped with autumn olive (*Elaeagnus umbellata* Thunb., Elaeagnaceae) (PJE stands), an alien species known as a N-fixing shrub given the association with symbionts capable of fixing N, used in tree farming plantations.

An adjacent agricultural field (AL stands) was also included in this study for comparison. Five topsoils (0–10 cm of depth) were collected using a core sampler in each replicate of the four trees associations and in the agricultural field in May 2018, resulting in 75 samples (i.e., 3 field replicates × 5 plots × 5 soil cores). This sampled depth was chosen to analyse the most active layer (Zhao et al., 2021). More details about the study site and the sampling design are reported elsewhere (Danise et al., 2021a).

Once in the laboratory, soil samples to be used for enzymatic analyses were stored at −80 °C, whereas samples for metabarcoding analyses were stored at 4 °C until analysis. The residual soil was air-dried and 2-mm sieved for further analyses.

2.2. Available phosphorous determination

Available phosphorus (AP) was determined according to Olsen method (Olsen and Sommers, 1982). For each sample, an extraction lasting 30 min was performed using a solution of sodium bicarbonate followed by molybdenum blue colorimetry. Absorbance was read on a UV spectrophotometer at 882 nm.

2.3. Cellulose determination

Spectrophotometric cellulose was measured following the Updegraff method (Bauer and Ibáñez, 2014) with minor modifications. Aliquots of 30–180 mg of dry soil, defined according to the total organic carbon (TOC) content, were put into 15 mL polypropylene tubes, and 3 mL of 8:2:1 (v/v/v) glacial acetic acid, distilled water, and nitric acid (69 %) solution were added. Samples were put into a thermostatic water bath at 90 °C for 30 min. Once removed hemicellulose and lignin, samples were treated with 2.5 mL 72 % sulphuric acid, and 1 mL of 2 g L^{-1} anthrone (dissolved in concentrated sulphuric acid) was added. Colorimetric determination was carried out with an UV-spectrophotometer at 690 nm against blank samples. The anthrone–furfural complex extinction coefficient ($\varepsilon = 36.00\ M^{-1}\ cm^{-1}$) was determined from a calibration curve.

2.4. Fluorescein diacetate hydrolase activity assay

Fluorescein diacetate hydrolase (FDAH) activity was determined spectrophotometrically according to Green et al. (2006) and tested in each stand on 5 field cores and 3 laboratory replicates.

An aliquot of soil was put in centrifuge tube and sodium phosphate buffer solution 60 mM pH 7.6 and a fluorescein diacetate substrate solution was added. Samples were incubated in a water bath at 37 °C for 3 h. Following the incubation, acetone 100 % was added, and samples analysed using an UV–VIS spectrophotometer at 490 nm. Enzyme activity was reported as μmol of product developed in one hour per gram of dry matter.

2.5. ITS2 metabarcoding

To characterize the fungal community in each soil sample, the internal transcribed spacer 2 (ITS2) of the rRNA cistron was amplified by a metabarcoding approach. The analyses were carried out on a sample pool obtained by mixing, in each stand, 5 field replicates, for a total of 15 samples. Total DNA was extracted from 0.25 g of soil sample (dry weight) using the DNeasy PowerLyzer PowerSoil Kit (QIAGEN), following a sample homogenization using an UltraCool GeneReady Homogenizator (Life Real). The isolated DNA was quantified using a Qubit 3 Fluorometer (Invitrogen, Thermo Fisher Scientific) and the quality was checked with both a spectrophotometer NanoReady Touch (Life Real) and UVIdoc HD5 gel documentation system (UVITEC). Metabarcoding libraries were prepared by amplifying by PCR the ITS2 region with a modified set of ITS3 (5′-GCA TCG ATG AAG AAC GCA GC-3′) and ITS4 primers (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990), which included the Illumina adapters (forward primer + 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG-3′; and reverse primer + 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G-3′). Each PCR was carried out into a reaction volume of 40 μL consisting of: ca. 10 ng of DNA template, 2× Kodaq PCR MasterMix (Applied Biological Materials Inc., ABM), 0.25 μM of each primer, and water to volume. The reaction conditions were as follows: an initial denaturation step of 94 °C for 3 min and then 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. The final extension was performed at 72 °C for 5 min. A duplicate for each amplicon set was carried out for reproducibility purposes. A negative blank (no DNA) was employed. To limit possible genomic DNA contaminations in the MasterMix and in the water, a pre-treatment before the PCR was carried out using 2.5 U of Bsp143I (*Sau*3AI) (Thermo Fisher Scientific) at 37 °C for 30 min (plus 65 °C for 20 min for enzyme inactivation) (Carroll et al., 1999; De Castro et al., 2024). A positive sample was carried out using a plasmid pGem-3Zf + (Applied Biosystem, Thermo Fisher Scientific) with the cut site for enzyme. The amplicons were checked using the UVIdoc HD5 Gel documentation system and quantified using a Qubit 3 Fluorometer. A volume of 25 μL (> 5 ng μL^{-1}) of each library was sent to BMR Genomics s.r.l. (Padua, Italy) for high-throughput sequencing using the Illumina MiSeq platform (300 bp paired-end sequencing; 2 × 50,000 reads/sample).

Illumina paired-ends reads were processed to generate amplicon sequence variants (ASVs) in Mothur v1.33.0 (Schloss et al., 2009). Primer sequences were removed and no ambiguous bases were allowed; maximum homopolymer size was set to 8 bp, minimum and maximum amplicon length to 250 and 450 bp, respectively. The remaining sequences were de-replicated and the *pre.cluster* function (default settings)

was used to generate ASVs. Putative chimeras were removed using vsearch (Rognes et al., 2016). Taxonomic assignment was carried out using the BLAST algorithm (Altschul et al., 1990) against the UNITE database (Nilsson et al., 2019) using an identity threshold of 90 %. Hits matching the reference sequences with a coverage <75 % of the minimum amplicon length (175 bp out of 250 bp) were discarded, as well as sequences not assigned to fungi. For a more clear and explicative fungi community description, the most representative taxa among all sites were selected. Thus, a threshold of 1 % was set based on the percentage abundance of each family per sample to obtain a Grand Weighted Mean (GWM) (Parisi et al., 2021).

2.6. Functional group analysis

To identify different functional groups within fungal communities and link their relative abundance to a specific stand, FunGuild (Nguyen et al., 2016a), an open annotation tool to assign fungal taxa into three ecologically relevant trophic modes, i.e., saprotrophy, symbiotrophy and pathotrophy, was used. These modes were further subdivided into specific guilds comprised of fungi that share similar lifestyle modes.

2.7. Statistics

Shapiro-Wilk test and Q-Q plots were used to check data normality, whereas Levene's test was run to test homogeneity of variance. Chi-squaredness was also evaluated.

One-way analysis of variance (ANOVA) was used to test the effects of the different stands on the response variables, followed by Tukey's post-hoc test ($\alpha = 0.05$). We used Two-Block Partial Least Squares (2B-PLS) to examine patterns of covariance between soil chemical and biological data (i.e., FDAH enzyme activity, AP, TOC, and total N) and fungal guilds. When dealing with matrices that have a small sample size as well as highly correlated variables, this statistical method can produce accurate inference (Barker and Rayens, 2003; Carrascal et al., 2009). This technique has recently been applied in several ecological contexts (Danise et al., 2022). A scatterplot for the first axis of the 2B-PLS, with the two multivariate matrices represented by the x-axis and the y-axis, respectively, was used to visualise patterns of covariance between the two matrices. It is possible to show patterns of inverse or positive correlation both within and between matrices. All statistical analyses were carried out using R (version 4.2.1; R Core Team, 2022).

3. Results and discussion

3.1. Soil chemical parameters

The absence of litter and the consequent lack in TOC input in the agricultural soil inevitably induces a change in soil chemical and physical conditions (Table 1), as also reported by de Graaff et al. (2019).

Compared to all afforested stands, AL presented the lowest values for all considered parameters except for AP, which is comparable to all the other stands (Table 1). Cellulose was less abundant than lignin, confirming that cellulose has a faster turnover (Chen et al., 2018). Cellulose serves as a very efficient energy source, while lignin is decomposed rather co-metabolically, requiring an easily available carbon (C) source (Berg and McClaugherty, 2014). Further, in fertilized agricultural soil, cellulose and lignin decomposition is significantly accelerated (Song et al., 2022). Consistently, Man et al. (2021) showed fertilization to alter the SOM components proportions with distinct biogeochemical cycling patterns and turnover rates. This has the potential to significantly influence SOM sequestration and dynamics. Man et al. (2021) observed a decrease in plant-derived steroids, cutin- and suberin-derived compounds suggesting enhanced degradation of these SOM components under N fertilization. In contrast, the enhanced lignin degradation appeared to be regulated by a combination of degradation processes, litter quantity, and litter quality. Taken together, these findings suggest that microbial degradation, along with the quality and quantity of crop residues, are likely the primary mechanisms governing SOM composition and cycling under fertilization regimes.

Obtained results also suggest that, despite the considerable mixed plantations potential in improving arable soil fertility and quality compared to monocultures (Liu et al., 2018), a natural forest structure induces a greater accumulation of SOM (Thomas et al., 2020). Among afforested plots, PJA showed highest concentrations of all parameters except for AP, since Italian alder litter is rapidly decomposed favouring C and N release into the soil (Innangi et al., 2017). Although most of SOM in forest soils is generally found in a particulate form (Cotrufo et al., 2019; Kooch et al., 2012), the higher clay content (200 ± 20 g kg^{-1}) in PJA stand soil (Danise et al., 2021a) could also suggest a relatively greater mineral protection of SOM against microbial degradation. PJE showed the lowest TOC and cellulose content (14.5 ± 0.8 and 0.9 ± 0.1 mg g^{-1}, respectively) which could be explained by the higher productivity, and consequently greater nutrient uptake of the valuable poplar and walnut species (Danise et al., 2021a). Furthermore, in line with literature (Zheng et al., 2021), AL stands showed lower C/P and N/P ratios compared to tree stands, possibly due to the lower crop residue inputs and the faster SOM decomposition.

3.2. Fluorescein diacetate hydrolase activity

Soil microbial activity was assessed by using FDAH enzyme activity. Deep differences emerged between the studied stands (Fig. 1).

The agricultural field (AL) showed lower FDAH activity compared to afforested stands, while PJA had the greatest activity with respect to both AL and other afforested stands. Few difference between PJ and PJE stands was found while PJC stands had a behaviour comparable to AL. FDAH results in AL are in agreement with findings of Rankoth et al. (2019) for a corn (*Zea mays* L.)–soybean (*Glycine max* [L.] Merr.) rotation after four years, while they showed higher values than those reported by Piotrowska-Długosz et al. (2022) for the surface soil of *Medicago sativa* L. agricultural fields.

Hence, agricultural management induced not only a decrease in TOC (Table 1) but also in microbial activity (Fig. 2) compared with forest management. Obtained results are in agreement with Martí-Roura et al. (2019) who reported that land use type greatly influenced soil chemical and biological characteristics. Long-term trees in forest ecosystems can continuously supply litter, enhance root turnover, keep or increase SOM content, and affect the activity of soil microbial organisms (Soleimani

Table 1
Soil chemical parameters (lignin, cellulose, available phosphorous (AP), total nitrogen (TN), total organic carbon (TOC) and relative ratios) of both afforested and the arable land stands (§data from Danise et al., 2020). Values represent mean ± standard error. Superscript letters indicate significant differences within means in the column according to One-way ANOVA at $p \leq 0.05$ (Supplementary Table 1) and Tukey test.

	Lignin§ (mg g^{-1})	Cellulose (mg g^{-1})	AP (mg kg^{-1})	TN§ (mg g^{-1})	TOC§ (mg g^{-1})	TOC/TN	TOC/AP	TN/AP
AL	3.40 ± 0.7^{c}	0.64 ± 0.0^{b}	10.47 ± 0.3^{a}	0.84 ± 0.0^{c}	8.28 ± 0.2^{c}	10.07 ± 0.4^{a}	0.70 ± 0.0^{b}	0.07 ± 0.0^{c}
PJ	5.68 ± 0.6^{b}	1.20 ± 0.1^{a}	10.91 ± 1.1^{a}	1.50 ± 0.1^{ab}	16.36 ± 0.8^{ab}	10.98 ± 0.4^{a}	1.51 ± 0.1^{a}	0.14 ± 0.0^{ab}
PJA	11.25 ± 1.1^{a}	1.54 ± 0.2^{a}	8.99 ± 1.2^{a}	1.78 ± 0.1^{a}	19.10 ± 1.8^{a}	10.55 ± 0.3^{a}	2.45 ± 0.3^{a}	0.23 ± 0.0^{a}
PJC	6.30 ± 0.5^{b}	1.09 ± 0.2^{ab}	12.22 ± 1.3^{a}	1.32 ± 0.1^{b}	17.43 ± 1.2^{ab}	13.39 ± 0.6^{b}	1.42 ± 0.1^{a}	0.11 ± 0.0^{b}
PJE	6.32 ± 0.4^{b}	0.91 ± 0.1^{ab}	8.95 ± 1.1^{a}	1.42 ± 0.1^{ab}	14.50 ± 0.8^{b}	10.15 ± 0.3^{a}	1.61 ± 0.2^{a}	0.16 ± 0.0^{a}

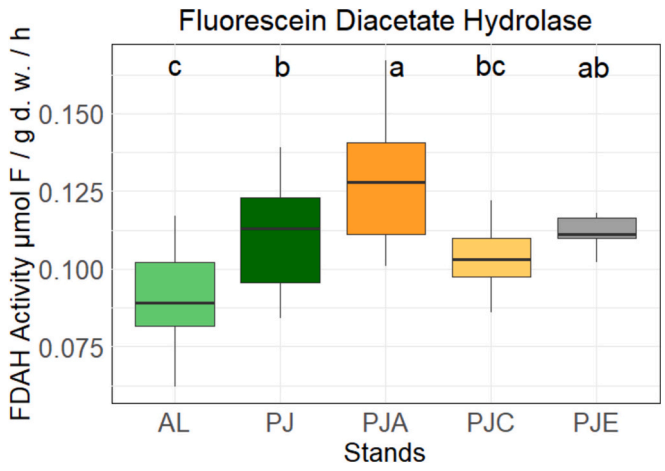

Fig. 1. Fuorescein diacetate hydrolase (FDAH) activity boxplot in afforested and arable land stands. Different superscript letters indicate significant differences ($p \leq 0.05$, Tukey's post-hoc test following One-way ANOVA).

et al., 2019). Our results obtained for PJA stands agree with those reported by Soleimani et al. (2019) who analysed soil C and microbial activity in several forest plantations such as *Alnus subcordata* C.A. Mey., *Acer velutinum* Boiss., *Quercus castaneifolia* C.A. Mey and *Cupressus sempervirens* L. plantations, compared to an agricultural field. Their findings confirmed that alder induces greater microbial activity as well as more organic C accumulation.

3.3. Metabarcoding results

The metabarcoding pipeline generated 191,950 ASVs; after removal of singletons, non-target sequences, and shortly annotated fragments, a final dataset of 11,695 ASVs with a mean amplicon length of 371 bp was obtained (Supplementary Table 2). The barplots of taxonomic composition of fungal communities at family level showed considerable differences depending on the stand (Fig. 2).

AL differed from other tree stands in terms of lower Basidiomycota abundance and higher Mortierellomycota occurrence. In agreement with Bastian et al. (2009), the most present phyla are Ascomycetes and Basidiomycetes in soils of the forested stands, while they showed only a small proportion of Mortierellomycota. One of the assumptions underlying this high global quantity is wind dispersion. However, Egidi et al. (2019) found that the globally dominant Basidiomycetes found in air samples do not belong to the soil dwelling Agaromycetes, whose members are often recovered in atmosphere. Therefore, wind dispersion is not sufficient to justify the massive presence of these phyla, but instead their lifestyle is a plausible reason. Members of Ascomycota have a reduced capability to decompose the recalcitrant lignin-containing litter material (Pérez-Izquierdo et al., 2021), thus they could use the easily degradable fraction of residues for fast-growing fungal populations. In fact, during SOM decomposition, labile components decrease, and more stable compounds accumulation would promote the related Basidiomycota decomposition activity. This could explain the absence of Basidiomycetes in AL, given the low amount of lignin and cellulose (Table 1), and the relatively high abundance of Ascomycetes. As shown in Figure 2, in PJ and PJA stands a similar Ascomycota and

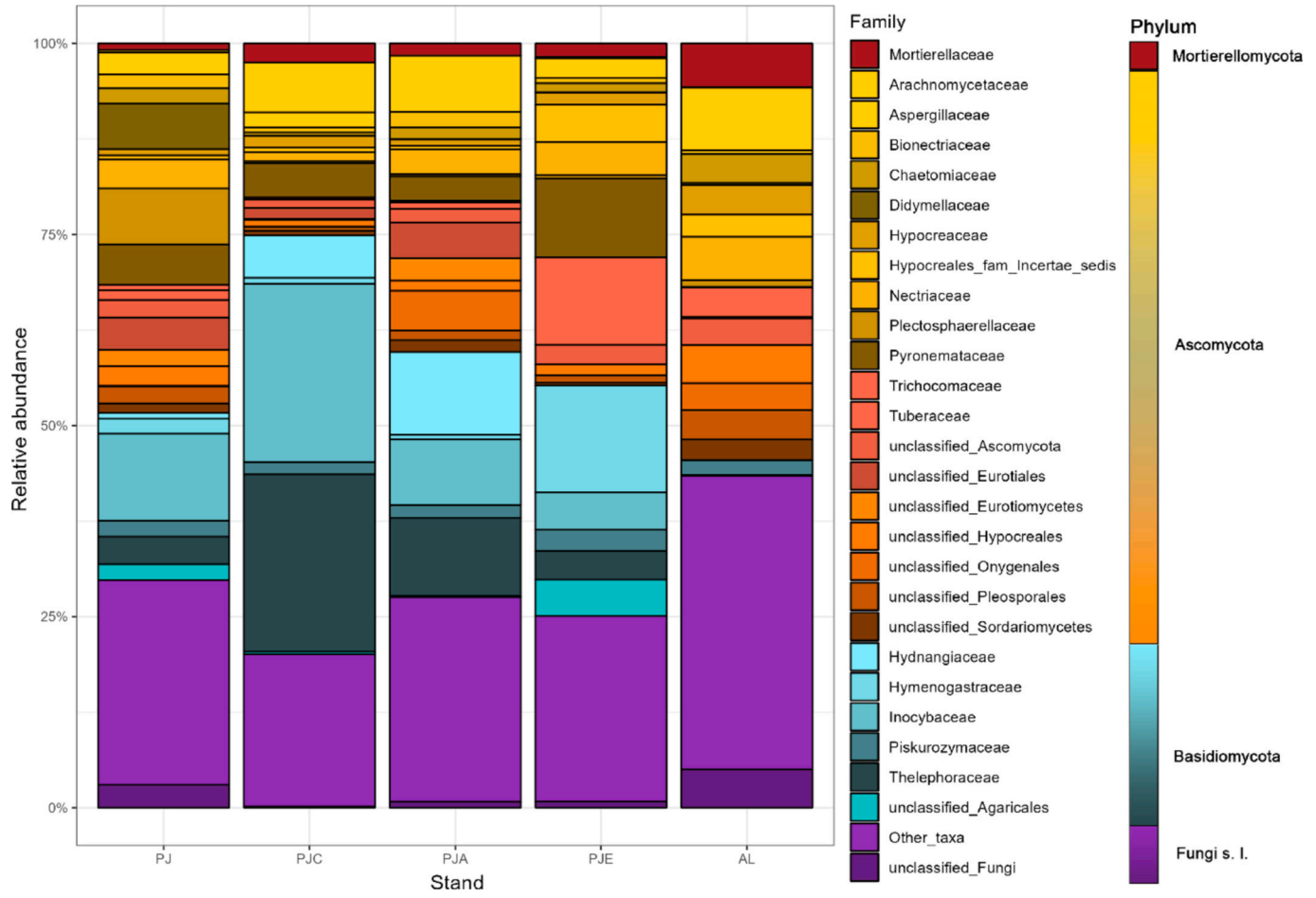

Fig. 2. Barplots of the most representative fungal families (and associated phyla) in both afforested and arable stands. Sensu lato (s. l.) refers to reads that could not be assigned to a specific taxonomic rank (i.e., unclassified Fungi) as well as families present in quantities less than the established threshold (i.e., other taxa).

Basidiomycota relative abundance was found but characterized by a different contribution of families belonging to respective phyla. A greater contribution of Chaetomiaceae, Didymellaceae, Plectosphaerellaceae, Pyronemataceae (Ascomycota), as well as Hymenogasteraceae (Basidiomycota) were found in PJ stand, whereas PJA stands had a higher Hydnangiaceae, Inocybaceae and Telephorace (Basidiomycota) abundance. PJE stands emerged for the higher Tuberaceae and Hymenogastraceae relative abundance. Tree stands that differed most from the others were PJC stands, being characterized by the lower Ascomycota relative abundance and the greatest of Basidiomycota. As reported in a previous study (Danise et al., 2020), PJC stands showed the lowest lignin-derived phenolic acid to their corresponding aldehydes ratios (Ac/Al) for syringyl units (Ac/Al)s. This ratio is generally used to characterize diagenetic alteration of SOM in a variety of geochemical samples (Ertel and Hedges, 1984; Zaccone et al., 2008). This could agree with the high Basidiomycetes quantity found in these stands, given their ability to hydrolase the more stable organic substances and completely decompose them, which results in a lower degradation state of the remnant lignin. In PJC stands, Inocybacee and Telephoracee showed the greater abundance among Basidiomycota families compared to the other afforested stands. To trace the microorganism functional role, the ecological guilds (Fig. 3) and the relative trophic groups were identified. Our results showed that the mainly represented trophic groups are symbiotrophs (PJA: 30.5 %, PJC: 53.5 %, PJE: 46.6 %, PJ: 25 %, AL: 0.2 %), followed by saprotrophs (PJA: 0.6 %, PJC: 10.8 %, PJE: 0.3 %, PJ: 2 %, AL: 5.3 %) and pathotrophs (PJA: 1.3 %, PJC: 0.5 %, PJE: 1.7 %, PJ: 1.4 %, AL: 0.8 %). Even if fungal genomes can present evidence of species guild associations (Zanne et al., 2020), in all stands, individuals who probably belong to different trophic groups and, consequently, to different guilds were found. For example, plant pathogen guild

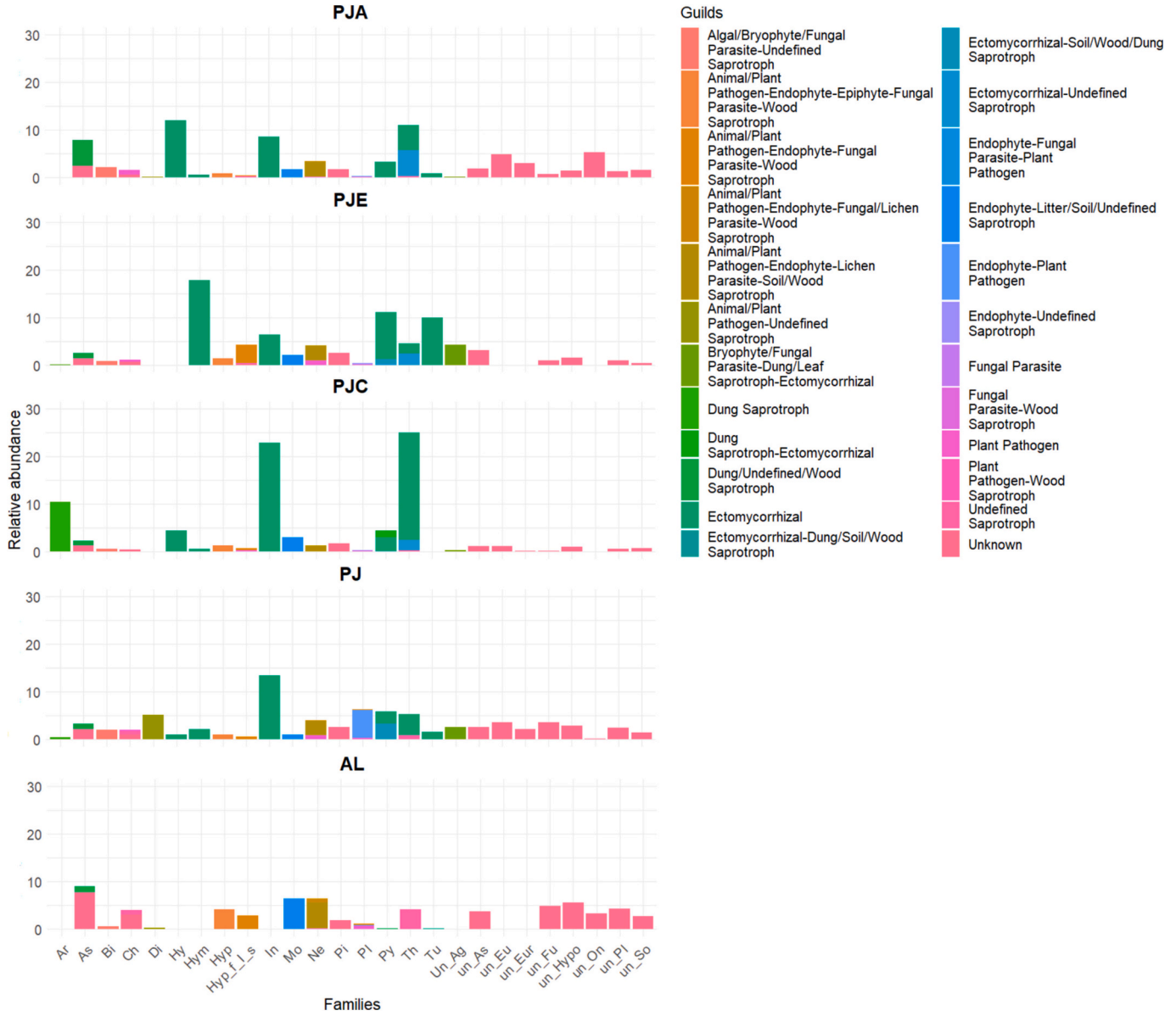

Fig. 3. Ecological guilds identified in each family in all stands. Only species found in quantities ≥ to the established threshold were represented. Families are indicated with following abbreviations: Arachnomycetaceae (Ar); Aspergillaceae (As); Bionectriaceae (Bi); Chaetomiaceae (Ch); Didymellaceae (Di); Hydnangiaceae (Hy); Hymenogastraceae (Hym); Hypocreaceae (Hyp); Hypocreales_fam_Incertae_sedis (Hyp_f_I_s); Inocybaceae (In); Mortierellaceae (Mo); Nectriaceae (Ne); Piskurozymaceae (Pi); Plectosphaerellaceae (Pl); Pyronemataceae (Py); Thelephoraceae (Th); Tuberaceae (Tu); unclassified_Agaricales (Un_Ag); unclassified_Ascomycota (Un_As); unclassified_Eurotiales (Un_Eu); unclassified_Eurotiomycetes (Un_Eur); unclassified_Fungi (Un_Fu); unclassified_Hypocreales (Un_Hypo); unclassified_Onygenales (Un_On); unclassified_Pleosporales (Un_Pl); unclassified_Sordariomycetes (Un_So).

components have high copy numbers of genes coding for plant carbohydrate-active enzymes (CAZys) (Kohler et al., 2015), widely present in saprotrophic fungi genomes because of they depend on dead plant matter decomposition for their C supply (Kohler et al., 2015). This suggests that most of fungi identified as plant pathogens follow a more necrotrophic than biotrophic strategy (Spanu et al., 2010).

Moreover, it is increasingly recognized that some necrotrophic fungi infect many host plant species asymptomatically as endophytes as well as causing disease in other hosts (Zanne et al., 2020). Thus, a deeper comprehension of coordination among traits, such as the presence, copy number and expression levels of genes coding for such enzymes (Gazis et al., 2016) could describe when, why, and how specific hosts and environment activate a pathogenic versus endophytic or saprotrophic lifestyle. This kind of taxa is copious in AL stands (Fig. 3), where Hypocreaceae family is characterized by Animal/Plant Pathogen - Endophyte - Fungal Parasite - Wood Saprotroph guild, Mortierellaceae family presented Endophyte - Litter/Soil/Undefined Saprotroph guild whereas Nectriaceae family showed high Animal/Plant Pathogen - Endophyte - Lichen Parasite - Soil/Wood Saprotroph guild relative abundance values. Furthermore, Saprotroph guild was represented in Thelephoraceae, Chaetomiaceae and Aspergillaceae family. These results are consistent with previous studies, which revealed that N fertilizer, which are normally applied in arable lands, could inhibit the growth of mycorrhizal fungi, and led to a shift in the fungal community dominance from mycorrhizal fungi to saprotrophic fungi (Ning et al., 2021).

In all tree stands, the most representative guild was the ectomycorrhizal one, since it was found in PJA stands (belonging to Hydnangiaceae, Inocybaceae and Pyronemataceae families), in PJC stands (in Hydnangiaceae, Inocybaceae and Thelephoraceae families), in PJE stands (in Hymenogastraceae and Tuberaceae families) and PJ stands (identified in Inocybaceae, Pyronemataceae and Thelephoraceae families). Saprophytes guild characterized Aspergillaceae families in PJA stands, Arachnomycetaceae families in PJC stands, few individuals were identified in PJE stands belonging to Aspergillaceae family, and in Thelephoraceae and Chaetomiaceae family in PJ stands. Plant pathogen guild belonging to Plectosphaerellaceae and Nectriaceae families was found in PJ stands. Thus, microbial community as well as soil chemical characteristics changed according to different soil managements.

3.4. Interactions between parameters

As shown by 2B-PLS (Fig. 4), within Block 1, a strong inverse correlation emerged between G11 (Ectomycorrhizal), G14

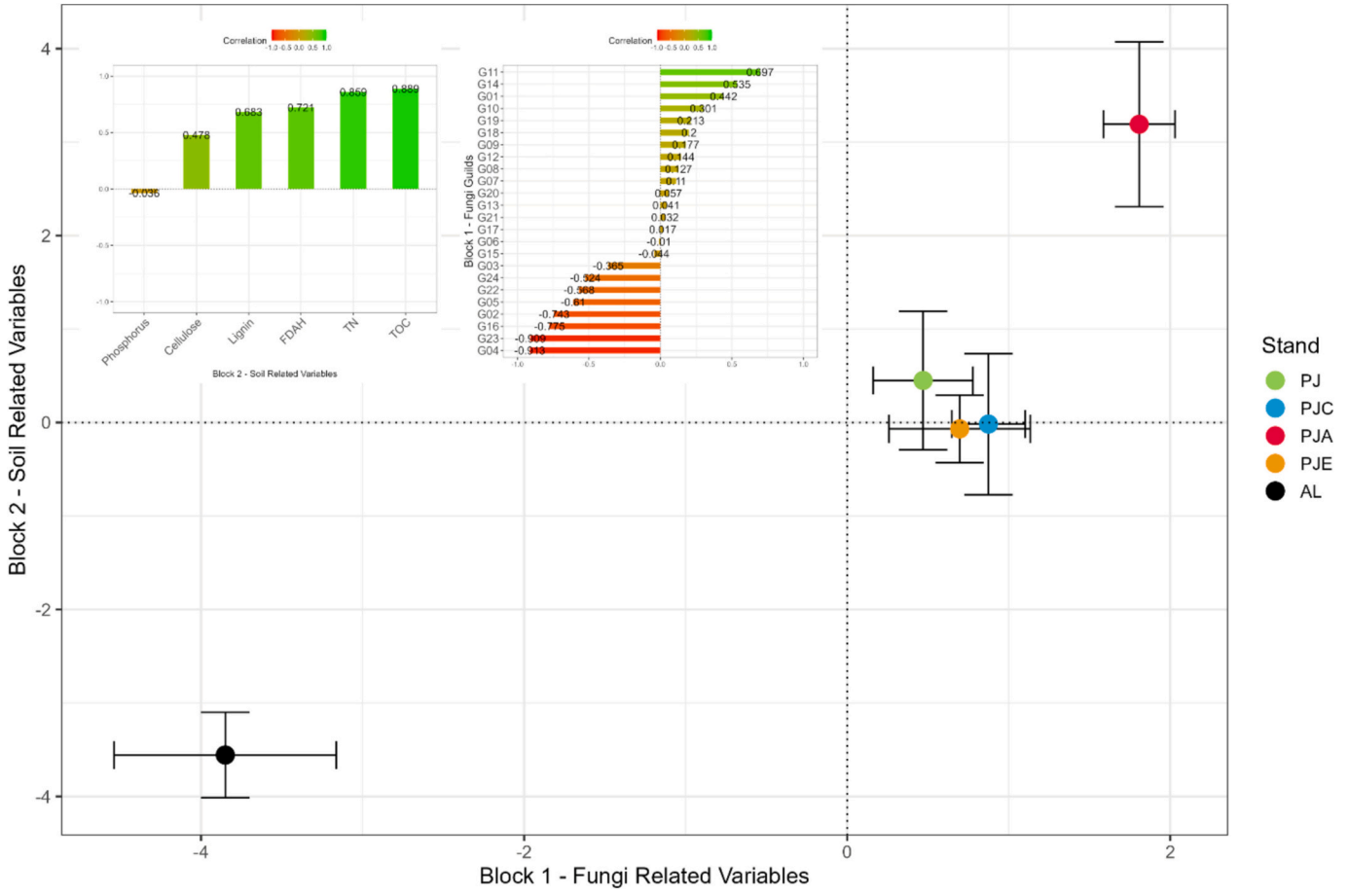

Fig. 4. Scatterplot for the first axis of the Two-Block Partial Least Squares. Points are mean ± standard error across Block 1 (Fungi related variables) and Block 2 (Soil related variables). The insets show the correlation within and between blocks of the variables.
Guilds legend: G01: Algal/Bryophyte/Fungal Parasite - Undefined Saprotroph; G02: Animal/Plant Pathogen - Endophyte-Epiphyte - Fungal Parasite - Wood Saprotroph; G03: Animal/Plant Pathogen - Endophyte - Fungal Parasite - Wood Saprotroph; G04: Animal/Plant Pathogen - Endophyte - Fungal/Lichen Parasite - Wood Saprotroph; G05: Animal/Plant Pathogen-Endophyte - Lichen Parasite - Soil/Wood Saprotroph; G06: Animal/Plant Pathogen - Undefined Saprotroph; G07: Bryophyte/Fungal Parasite-Dung/Leaf Saprotroph - Ectomycorrhizal; G08: Dung Saprotroph; G09: Dung Saprotroph - Ectomycorrhizal; G10: Dung/Undefined/Wood Saprotroph; G11: Ectomycorrhizal; G12: Ectomycorrhizal -Dung/Soil/Wood Saprotroph; G13: Ectomycorrhizal - Soil/Wood/Dung Saprotroph; G14: Ectomycorrhizal - Undefined Saprotroph; G15: Endophyte - Fungal Parasite - Plant Pathogen; G16: Endophyte - Litter/Soil/Undefined Saprotroph; G17: Endophyte - Plant Pathogen; G18: Endophyte - Undefined Saprotroph; G19: Fungal Parasite; G20: Fungal Parasite - Wood Saprotroph; G21: Plant Pathogen; G22: Plant Pathogen - Wood Saprotroph; G23: Undefined Saprotroph; G24: Unknown.

(Ectomycorrhizal - Undefined Saprotroph), G01 (Algal/Bryophyte/Fungal Parasite - Undefined Saprotroph), and G16 (Endophyte - Litter/Soil/Undefined Saprotroph), G23 (Undefined Saprotroph), G04 (Animal/Plant Pathogen - Endophyte - Fungal/Lichen Parasite - Wood Saprotroph).

Thus, overall, there was an inverse correlation between Ectomycorrhizal (EM) and Saprotroph guilds. It could be explained considering that EM may modulate SOM storage by removing organic N, the so called 'N-mining' hypothesis, whereas EM fungi are supposed to oxidize SOM to obtain small organic N-bearing compounds leaving relatively C-rich substrates behind (Averill and Hawkes, 2016). It implies nutrient restriction for the residual free-living saprotrophic community and develops in the so-called Gadgil effect (Zak et al., 2019). Numerous studies demonstrated EM fungi competing with saprotrophic fungi for resources in pure cultures or microcosms (Wu et al., 2003). Fewer studies, however, have explored these interaction effects on litter or SOM decomposition rates. Gadgil and Gadgil (1975) complemented their field study with a microcosm experiment involving saprotrophic fungi and both EM and non-EM colonized plants, which widely corroborated the competitive exclusion process. It could also explain the strong direct correlation among G11 (Ectomycorrhizal), G14 (Ectomycorrhizal-Undefined Saprotroph) and all soil related variables found between the blocks, whereas a decrease of SOM decomposition by saprotrophs induces soil C accumulation. Furthermore, EM fungi may directly utilize nutrients found in the biomass of saprotrophic fungi, which may lead to the suppression of litter and SOM decomposition processes (Fernandez and Kennedy, 2016).

The second block shows that all biological and chemical variables (cellulose, lignin, FDAH, TN, TOC) are positively correlated with each other. Stands that are placed in correspondence with positive values along first block axis (x) are characterized by a high Ectomycorrhizal and Ectomycorrhizal-Undefined Saprotroph guild relative abundance, while those located in correspondence with negative values emerge above all for Saprotroph and Animal/Plant Pathogen - Endophyte - Fungal/Lichen Parasite - Wood Saprotroph guilds presence (Fig. 4). Similarly, the stands that are in correspondence with second block axis (y) positive values are distinguished by high biological and chemical soil related variables amount. Therefore, a clear pattern emerged where AL is distinguished by a copious presence of Saprotroph guilds and a scarce quantity of all considered soil biological and chemical variables (except AP) while, among the forested stands, PJA stands were characterized mainly by high values of their biological and chemical variables in concomitance with the higher Ectomycorrhizal guild presence. The other tree stands had an intermediate position. The relative increase in saprotrophs and decrease in symbiotrophs under standard tillage (AL) was associated with reduced SOM and nutrient content, in line with the classic C decomposition and loss model primarily regulated by fungal saprotrophs. Ning et al. (2021) showed that the N/P ratio is a very useful indicator in explaining the shift of saprotrophic fungal communities. Soil N/P ratios are lower in agricultural soil than in forest soils (Table 1), resulting in N-limited conditions and suggesting the dominance of specific microorganisms capable of mineralizing N from SOM. Also, the C/P ratio was smallest in AL indicating, together with the small N/P ratio, that P was not deficient and showed that saprotrophic fungi are able to improve soil P availability by, for example, solubilization of phosphate via the release of organic acids, and synthesis of specific enzymes (Ceci et al., 2018). Among tree stands, PJC stood out for the higher C/N ratio and lower N/P ratio (Table 1). The greater presence of EM fungi in these stands (Fig. 3) confirms their role in oxidizing SOM to obtain organic N-bearing compounds in N-limited conditions.

Recent research demonstrated that soil saprotrophic fungal communities change along soil fertility gradients in natural ecosystems, such as forests and grasslands (Chen et al., 2020). Accordingly, PJA stands presented the opposite condition compared to the fertilized agricultural field. Furthermore, in agreement with Ardanuy et al. (2021), alder association with N-fixing bacteria *Frankia* in PJA stands relieved N limitation and, as shown by the high N/P and C/P ratios, likely increased plant P demand (Table 1), which may promote the formation of ectomycorrhizas to mitigate this need. Despite including also a N-fixing species and being characterized by a discrete EM abundance, PJE stands had lower C and N concentrations respect to PJA stands. As shown in Danise et al. (2021a), *Elaeagnus umbellata* improved valuable species growth by increasing plant nutrient uptake and, consequently, depleted soil C and N reserves. Furthermore, in the PJE stands, the green leaves had a different metabolic profile compared to the tree species of the other stands (Danise et al., 2021b), suggesting an allelochemical mechanism probably mediated just by EM in symbiosis with the *Elaeagnus umbellata*. Consistently, previous studies (Hicks Pries et al., 2023; Zhang et al., 2021) showed that plant communities, protected by nurse species, alleviated local abiotic stress and promoted plant-microbe interactions, both through biomass and biodiversity effects. These findings shift the concept of nurse species from mere beneficiaries of nurturing effects to co-leaders of essential ecosystem functions. On the other hand, Innangi et al. (2017) showed that Italian alder leaf litter has low lignin and high ethanol extractable contents, which promote a fast litter decomposition rate and, consequently, a high soil TOC quantity that can build up as microbial C. Underlying alder benefits in mixed plantations may be multiple mechanisms that would require further investigation. Tight interactions between roots and microorganisms have been reported for example by Zhang et al. (2022), who investigated in situ root patterns, defence chemicals, microbial communities, and biochemical rhizosphere properties in soils from mixed *Eucalyptus grandis* W. Hill ex Maiden with *Alnus formosana* (Burkill) Makino stands. They concluded that belowground ecological facilitative interactions occurred in mixed plantations with alder, which were due to *E. grandis* altering its rooting pattern, reducing the levels of released allelochemicals, recruiting more beneficial bacteria, and improving the biochemical properties as well as rhizosphere soil biodiversity.

4. Conclusions

Soil chemical and biological features differ depending on intercropping. When compared to the agricultural area, all forest stands show significantly higher concentrations of TOC, TN, lignin, and cellulose, as well as in FDAH activity. Metabarcoding analyses revealed that forest stands are primarily characterized by EM fungi, whereas AL is primarily characterized by saprophytes. This may suggest a competition mechanism between these two guilds in which ectomycorrhizae release low-N compounds following SOM mineralization and create an unfavourable environment for saprophytes. Thus, in the presence of tree species, EM symbionts outcompete saprophytes. The low C/P and N/P ratios reported in AL, on the other hand, validate both the high N mineralization (most likely by saprophytes themselves) and the sufficient P quantity hypothesis, confirming the ability of saprophytes to make P bioavailable. Among the forested stands, PJA had the highest values of all chemical and biological parameters except AP, as well as an elevated level of EM. We conclude that since the known alder symbiosis with the genus *Frankia* causes a P shortage, EM may alleviate this requirement. Thus, mixed plantation is a tool to improve the health of marginal soils, whereas nurse species can have specific effects not only on involved plant species, but also on belowground compartment. In particular, in a mixed species plantation context, *Alnus cordata* could represent a plus improving both chemical and biological soil characteristics.

As this study was conducted at a single Mediterranean site, the results cannot be reliably extended to other biomes. The experiment should be repeated in a different set of bioclimatic conditions in order to allow for a generalisation of the results.

CRediT authorship contribution statement

Tiziana Danise: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation,

Conceptualization. **Olga De Castro:** Writing – review & editing, Formal analysis, Data curation. **Claudio Zaccone:** Writing – review & editing, Supervision. **Georg Guggenberger:** Writing – review & editing, Formal analysis. **Cristina Menta:** Writing – review & editing. **Michele Innangi:** Writing – review & editing, Formal analysis, Data curation. **Daniele De Luca:** Writing – review & editing, Formal analysis, Data curation. **Emanuela Di Iorio:** Writing – review & editing, Formal analysis. **Benedetta Turchetti:** Writing – review & editing. **Antonietta Fioretto:** Writing – review & editing, Supervision, Investigation, Funding acquisition.

Declaration of competing interest

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

Appendix A. Supplementary data

Raw metabarcoding data are available in the NCBI Sequence Read Archive (SRA) under BioProject PRJNA1010563. Supplementary Table 2 is available on figshare at https://figshare.com/s/794829eb660b54ffe9a5 and doi: https://doi.org/10.6084/m9.figshare.24511789. Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.105892.

Data availability

Data will be made available on request.

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