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Black truffle (Tuber melanosporum Vittad.) interactions with soil and plants in productive systems, and associated ecosystem services

Vasiliki Barou

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DISSERTATION
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by

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Summary

The main objective of the thesis is to study the abiotic and biotic interactions of the valuable edible fungus *Tuber melanosporum* Vittad. (black truffle) and their connections to soil functioning and ecosystem services in both wild and managed producing systems. For this, a regional field design with black truffle productive plantations and forests has been set up across the natural distribution area of the fungus in Spain (**Chapters 1** to **3**), a mesocosm experiment with dual cultures of truffle-oaks and medicinal and aromatic plants with native arbuscular mycorrhizal fungi has been established (**Chapter 4**), and a survey about the most salient ecosystem services associated to truffle-producing systems has been conducted (**Chapter 5**).

The abundance of black truffle mycelium is influenced by a combination of soil and climatic factors, with different variables driving mycelium growth in forests and plantations. However, the type of truffle-producing system does not affect the soil mycelium biomass (**Chapter 1**). The increase in black truffle abundance significantly reduces soil enzymatic activities related to carbon and nitrogen both in plantations and forests, underscoring its role in soil fertility and health (**Chapter 2**).

Soil fungal communities within black truffle brûlés in plantations are more homogeneous and exhibit a more complex fungal co-occurrence network compared to forests, suggesting greater stability likely due to the uniform conditions of the agroforestry systems. *Tuber melanosporum* does not act as a keystone species in soil fungal networks, although it likely has a more central role in forest than in plantations. A consistent regional pattern in nitrogen-related soil enzymatic activities linked to the saprotrophs has been observed in plantations, despite the strong local site effect on soil carbon and nutrient cycling (**Chapter 3**).

The co-culture of truffle oaks and medicinal and aromatic plants significantly influences the growth of plants and soil fungal symbionts in dual cultures. The presence of medicinal and aromatic plants negatively impacts the growth of truffle-oaks and *T. melanosporum* mycelium, especially when inoculated with native arbuscular mycorrhizal fungi that likely compete with *T. melanosporum* for colonisation of truffle-oak roots (**Chapter 4**).

Truffle-producing systems provide a range of ecosystem services, including provisioning, regulating and cultural ones. Further analysis of ecosystem services perception by stakeholders reveals that, among other factors, irrigation is recognised as a significant barrier for truffle cultivation sustainability. Moreover, truffle growers are interested in implementing secondary crops to generate

additional income and ecological benefits during the initial years of truffle orchard establishment (**Chapter 5**).

Overall, the findings obtained in this thesis contribute to a better understanding of black truffle ecology and highlight the importance of considering fungal and plant interactions, soil biodiversity and functioning, and ecosystem services to promote sustainable truffle management.

Resumen

El objetivo principal de la tesis es estudiar las interacciones abióticas y bióticas del hongo comestible y altamente apreciado *Tuber melanosporum* Vittad. (trufa negra), y sus conexiones con el funcionamiento del suelo y los servicios ecosistémicos en sistemas productores de trufa, tanto silvestres como gestionados. Para ello, se ha establecido un diseño experimental de campo a escala regional en plantaciones y bosques productores de trufa negra a lo largo del área de distribución natural del hongo en España (**Capítulos 1** a **3**), se ha realizado un estudio de mesocosmos con cultivos duales de encinas micorrizadas con trufa y plantas medicinales y aromáticas inoculadas con hongos micorrízicos arbusculares nativos (**Capítulo 4**), y se ha llevado a cabo una encuesta sobre los servicios ecosistémicos más destacados asociados a los sistemas productores de trufa (**Capítulo 5**).

La abundancia de micelio de la trufa negra está influenciada por una combinación de factores edáficos y climáticos, con diferentes variables impulsando el crecimiento del micelio en bosques y plantaciones. Sin embargo, el tipo de sistema productor de trufa no afecta directamente a la biomasa del micelio en el suelo (**Capítulo 1**). Una mayor abundancia de la trufa negra reduce significativamente las actividades enzimáticas del suelo relacionadas con el ciclado de carbono y nitrógeno, tanto en plantaciones como en bosques, subrayando su papel en la fertilidad y la salud del suelo (**Capítulo 2**).

Las comunidades fúngicas del suelo dentro de los brûlés de trufa negra en las plantaciones son más homogéneas y exhiben una red de co-ocurrencia fúngica más compleja en comparación con los bosques, lo que sugiere una mayor estabilidad, probablemente debido a las condiciones uniformes de los sistemas agroforestales. *Tuber melanosporum* no actúa como una especie clave en las redes fúngicas del suelo, aunque probablemente tiene un papel más central en los bosques que en las plantaciones. En plantaciones, se ha observado un patrón regional consistente en las actividades enzimáticas del suelo relacionadas con el ciclo del nitrógeno, vinculadas al grupo funcional de hongos saprófitos, a pesar del fuerte efecto local sobre el ciclado de carbono y nutrientes del suelo (**Capítulo 3**).

El co-cultivo de las encinas truferas y plantas medicinales y aromáticas influye significativamente en el crecimiento de las plantas y los simbiontes fúngicos del suelo en cultivos duales. La presencia de plantas medicinales y aromáticas impacta negativamente en el crecimiento de las encinas truferas y en el micelio de *T. melanosporum*, especialmente cuando se inoculan con hongos micorrízicos arbusculares nativos que probablemente compiten con *T. melanosporum* por la colonización de las raíces de las encinas (**Capítulo 4**).

Los sistemas productores de trufa proporcionan una variedad de servicios ecosistémicos, incluidos los de provisión, regulación y culturales. Un análisis más profundo de la percepción de los servicios ecosistémicos por parte de los actores principales del sector revela que, entre otros factores, el riego se reconoce como una barrera significativa para la sostenibilidad del cultivo de trufas. Además, los productores de trufas están interesados en implementar cultivos secundarios para generar ingresos adicionales y beneficios ecológicos durante los primeros años de establecimiento de una plantación trufera (**Capítulo 5**).

En general, los resultados obtenidos en esta tesis contribuyen a una mejor comprensión de la ecología de la trufa negra y resaltan la importancia de considerar las interacciones entre hongos y plantas, la biodiversidad y funcionalidad del suelo, así como los servicios ecosistémicos para promover una gestión sostenible de la trufa negra.

Resum

L'objectiu principal d'aquesta tesi és estudiar les interaccions abiòtiques i biòtiques del fong comestible i altament apreciat *Tuber melanosporum* Vittad. (tòfona negra) i les seves connexions amb el funcionament del sòl i els serveis ecosistèmics en sistemes productius de tòfona, tant silvestres com gestionats. Per a això, s'ha establert un disseny experimental en plantacions i boscos productors de tòfona negra al llarg de la distribució natural del fong a Espanya (**Capítols 1** a **3**), s'ha realitzat un estudi de mesocosmos amb cultius duals de alzines micorrizades amb tòfona i plantes medicinals i aromàtiques inoculades amb fongs micorrízics arbusculars nadius (**Capítol 4**), i s'ha dut a terme una enquesta sobre els serveis ecosistèmics més destacats associats als sistemes productors de tòfona (**Capítol 5**).

L'abundància de miceli de la tòfona negra està influenciada per una combinació de factors edàfics i climàtics, amb diferents variables impulsant el creixement del miceli en boscos i plantacions. No obstant això, el tipus de sistema productiu de tòfona no afecta directament la biomassa del miceli al sòl (**Capítol 1**). L'augment de l'abundància de la tòfona negra redueix significativament les activitats enzimàtiques del sòl relacionades amb el carboni i el nitrogen tant en plantacions com en boscos, subratllant el seu paper en la fertilitat i en la salut del sòl (**Capítol 2**).

Les comunitats fúngiques del sòl dins dels brûlés de tòfona negra a les plantacions són més homogènies i exhibeixen una xarxa de co-ocurrència fúngica més complexa en comparació amb els boscos, la qual cosa suggereix una major estabilitat, probablement a causa de les condicions uniformes dels sistemes agroforestals. *Tuber melanosporum* no actua com una espècie clau en les xarxes fúngiques del sòl, tot i que probablement té un paper més central en els boscos que en les plantacions. En plantacions, s'han observat patrons regionals consistents en las activitats enzimàtiques del sòl relacionades amb el nitrogen, impulsats pel grup funcional de fongs saprofítics, malgrat els forts efectes locals, sobre el reciclat de carboni i nutrients del sòl (**Capítol 3**).

El co-cultiu de alzines tofoneres i plantes medicinals i aromàtiques influeix significativament en el creixement de les plantes i en els simbionts fúngics del sòl en cultius duals. La presència de plantes medicinals i aromàtiques impacta negativament en el creixement de les alzines trufers i en el miceli de *T. melanosporum*, especialment quan s'inocula amb fongs micorrízics arbusculars nadius que probablement competeixen amb *T. melanosporum* per la colonització de les arrels de les alzines tofoneres (**Capítol 4**).

Els sistemes productors de tòfona proporcionen una varietat de serveis ecosistèmics, inclosos els d'aprovisionament, regulació i culturals. Una anàlisi més profunda de la percepció dels serveis ecosistèmics per part dels actors principals en el sector revela que, entre altres factors, el reg es reconeix com una barrera significativa per a la sostenibilitat del cultiu de tòfona. A més, els productors de tòfona estan interessats en implementar cultius secundaris per generar ingressos addicionals i beneficis ecològics durant els primers anys d'establiment d'una plantació tofonera (**Capítol 5**).

En general, els resultats obtinguts en aquesta tesi contribueixen a una millor comprensió de l'ecologia de la tòfona negra i ressalten la importància de considerar les interaccions fúngiques i amb plantes, la biodiversitat y funcionament del sòl, i els serveis ecosistèmics, per promoure una gestió sostenible de la tòfona negra.

General introduction

Economic importance of edible fungi and truffles

Humans have been collected edible mushrooms in the wild since ancient times for their high nutritional value, the pleasant organoleptic characteristics and the health benefits that they offer (Miles & Chang, 2004; Kaliyaperumal et al., 2018; Thu et al., 2020; Assemie & Abaya, 2022; Hamza et al., 2024). Given these properties and the increasing gastronomic interest in diverse key species, such as truffles and porcini (Kaliyaperumal et al., 2018), edible mushrooms have been cultivated with great success in recent years. According to FAOSTAT data (FAO, 2023), the global production of mushrooms and truffles has been constantly growing annually since 1960s', both in quantity of production and gross value (**Fig. 1**).

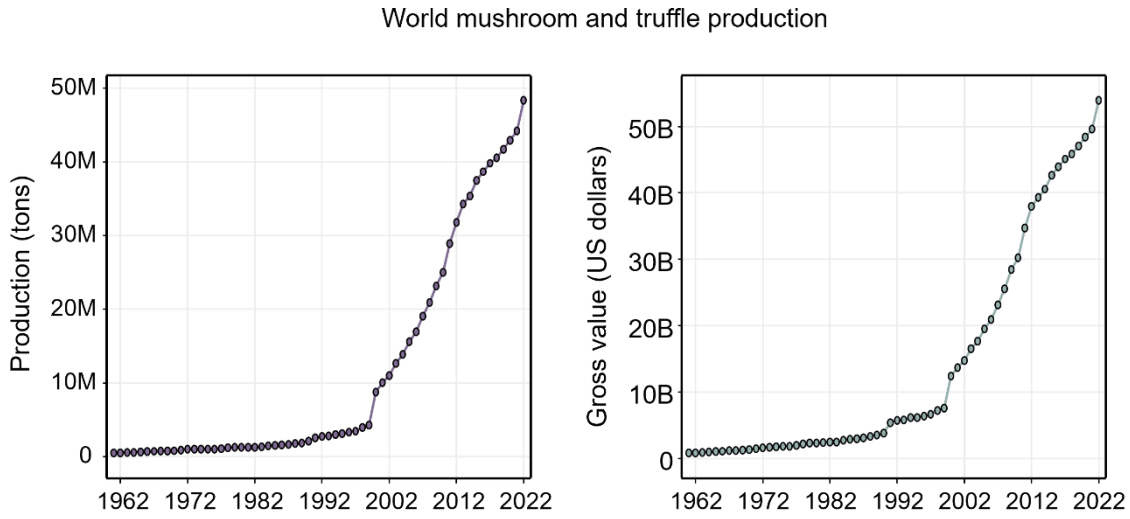


Figure 1 Global production of mushrooms and truffles worldwide through the years, according to FAOSTAT data (FAO, 2023).

Among edible fungi, truffles (*Tuber* spp.) are a group of species considered economically very important (Qin & Feng, 2022). Specifically, the black truffle (*Tuber melanosporum* Vittad.), the white truffle (*Tuber magnatum* Pico), the Burgundy truffle (*Tuber aestivum* Vittad.) and the *bianchetto* (*Tuber borchii* Vittad.) stand out for their aroma and flavor, and they exemplify the most valuable species of the genus (Iotti et al., 2016; Le Tacon et al., 2016). As a result of the growing gastronomic popularity, the economic potential, and the decline in wild populations, truffle production shifted from truffle hunting in the wild to truffle cultivation in plantations (Stobbe et al., 2013; Marozzi et al., 2017; Meadows et al., 2020; Zhang et al., 2020). The essential step for this shift was given during the

twentieth century with the successful truffle inoculation of host plants and, since early 1970s´, this technique started to improve (Murat, 2015). Nowadays, truffle trade represents a multimillion business worldwide (Lovrić et al., 2018, 2020) and, particularly in marginal areas, truffle cultivation offers an important economic resource (Donnini et al., 2013). In fact, black truffle is one of the most cultivated truffle species all over the world (Bonito & Smith, 2016; Oliach et al., 2021), and it has attracted the scientific attention to better understanding its biology, the interactions with the environment and its optimum cultivation techniques (Kües & Martin, 2011; Büntgen et al., 2017).

Spatial distribution

Species of genus *Tuber* have a large geographic distribution, appearing almost exclusively in the Northern Hemisphere, and specifically *T. melanosporum* is a typical species of Mediterranean countries like France, Italy and Spain (Le Tacon, 2016). However, the growing interest in truffle cultivation has led to the introduction of *T. melanosporum* into areas outside its natural distribution, from northern European countries, like Sweden, to South Africa, and from north and south America to Australia and New Zealand (Payen et al., 2014; Meadows et al., 2020; Tejedor-Calvo et al., 2023). Currently, Spain is considered one of the major producers of black truffle in the world (Herrero de Aza et al., 2022). Spanish forests naturally producing black truffle are mostly located at the eastern part of the country (Garcia-Barreda et al., 2019). Nevertheless, results of an experimental black truffle plantation at the central-western part (i.e., Salamanca province) showed a successful establishment of the mycorrhizal association and a satisfactory truffle yield, revealing the potential of truffle cultivation expansion in other regions of the Iberian Peninsula (Sánchez et al., 2018). In this sense, studying the fungus´ ecology across a range of truffle productive sites could contribute in identifying favourable environmental patterns and thereby expanding the black truffle cultivation.

Biology

Fungi, as heterotrophic organisms, get their energy from living or dead tissues of plant and animal origin. Fungi are categorised as saprophytic when their source of energy is dead organic material, parasitic when they obtain energy from living organisms harming their hosts and, mutualistic fungi when they live in close symbiotic association with plants and/or animals, with whom they interchange vital benefits (Miles & Chang, 2004).

Numerous soil fungi form a mutualistic association with roots of vascular plants, the mycorrhizal symbiosis (Smith & Read, 2008; Strullu-Derrien et al., 2018), derived from the Greek “myko”, meaning fungus, and “rhiza”, standing for root. This mutualistic association is beneficial for both symbionts as

fungi are supplied with carbohydrates from roots that the plant produces through photosynthesis and, in turn, plants acquire nutrients and water that the fungal mycelia absorb from the soil and supply to the roots (Miles & Chang, 2004). Mycorrhizal associations are the rule in natural ecosystems with an approximate estimate of 50,000 fungal species and 250,000 plant species involved in this symbiosis (Van Der Heijden et al., 2015; Brundrett & Tedersoo, 2018).

The principal types of mycorrhizal symbioses according to their anatomy and evolutionary ecology are the arbuscular mycorrhizal (AM), ectomycorrhizal (ECM), ericoid mycorrhizal (ERM) and orchid mycorrhizal (OM) (Smith & Read, 2008; Van Der Heijden et al., 2015; Brundrett & Tedersoo, 2018). The black truffle (*Tuber melanosporum* Vittad., Ascomycota, Discomycetes, Pezizomycota, Pezizomycetes, Pezizales, Tuberaceae) (**Fig. 2**) is an ECM fungus, meaning that its mycelium forms dense hyphal mats that enwrap the root tips of the host plant (Jansa & Treseder, 2017) and penetrates between the root cortical cells forming the Hartig Net, where the bidirectional exchange of nutrients happens. The fungus is hosted in a range of deciduous tree species, mainly belonging to the genera of *Carpinus*, *Cedrus*, *Cistus*, *Corylus*, *Pinus*, *Populus*, *Quercus*, and *Tilia* (Gryndler, 2016).



Figure 2 Black truffle (*Tuber melanosporum* Vittad.).

Life cycle

The black truffle in a hypogeous fungus, exclusively living belowground. Its life cycle begins in late winter or early spring, when the haploid spores, released by mature ascocarps, germinate (Payen et al., 2014) (**Fig. 3**). The fungus is heterothallic, and the genes involved in the reproductive process are *MAT1-1-1* and *MAT1-2*-1 (Rubini, Belfiori, Riccioni, Tisserant, et al., 2011). The germinated spores produce primary mycelium and establish ectomycorrhizal symbiosis with the root tips of the host plants (Rubini, Belfiori, Riccioni, Arcioni, et al., 2011). Then, in late spring or early summer, the primordia of truffle ascocarps are formed and they grow slowly until autumn, when they start to mature (Payen et al., 2014). Finally, the harvest period of the mature black truffles in the Northern Hemisphere ranges from November to March of the following year (Payen et al., 2014; Le Tacon et al., 2016). Based on the seasonal dynamics of its biological cycle, the assessment of black truffle extraradical mycelium and mycorrhizas is crucial for the optimisation of truffle production (Queralt et al., 2017).

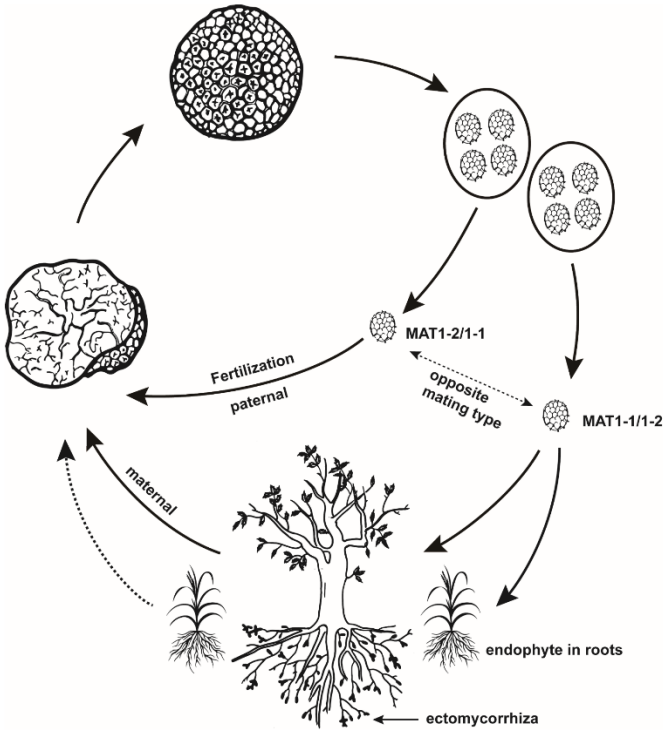


Figure 3 Life cycle of true truffles (Qin & Feng, 2022).

Truffle brûlé

The mycelium of black truffle produces substances that inhibit the growth of herbaceous plants in the area around the hosts, where the fungus is developed (González-Armada et al., 2010). This phytotoxic effect forms an area with reduced vegetation, as if the fungus was a natural herbicide (Olivera et al., 2014). Due to the scant vegetation, this area is called “burnt” or “brûlé” and makes the presence of black truffle in soil visible to the naked eye (Streiblová et al., 2012) (**Fig. 4**). Apart from the plant community, the black truffle does also affect soil fungal (Taschen et al., 2020) and bacterial communities (Mello et al., 2013). When the truffle mycelium occurs spontaneously in an area, the brûlé may promote the establishment of the fungus and/or the growth of truffle ascocarps, but there is still lack of evidence on the reasons of the brûlé formation (Schneider-Maunoury et al., 2020).



Figure 4 Black truffle brûlé represented by the circular area with scant vegetation around the trunk of colonised holm oak in Huesca, Spain.

Black truffle cultivation

The host plant

The interaction between the fungus and the host is critical for the former in order to produce mature fruiting bodies (ascocarps) and thus, to complete its life cycle (Payen et al., 2014). For truffle culture, host seedlings are inoculated with spores of *T. melanosporum* and, once the mycorrhizal

association has been established, they are planted in the field. The production of truffle-inoculated seedlings takes place mostly in commercial nurseries, resulting in a business of high economic importance (Donnini et al., 2014; Gómez-Molina et al., 2023). In Spain, seedlings of holm oak, *Quercus ilex* subsp. *ballota* (Desf.) Samp., are widely used to inoculate with *T. melanosporum* because of its durability, robustness and the higher truffle yields in comparison with other host species (Andrés-Alpuente et al., 2014).

Agricultural requirements

Once the host seedlings are colonised with *T. melanosporum* and planted in the field, black truffle production begins within 6-10 years, although the adequate agricultural practices are critical to ensure the success of truffle plantation (Bonet et al., 2009; Olivera et al., 2011). Indeed, after the establishment in the field and before the brûlé formation, both young seedlings and *T. melanosporum* may be affected by weed competition (Olivera et al., 2011). The control of the herbaceous cover, either mechanically or with the use of mulches, has been proved to enhance the growth of both symbionts (Olivera et al., 2014). In addition, a common practice in Spanish truffle orchards is the irrigation throughout the growing season (Garcia-Barreda et al., 2020). Medium intensity irrigation during summer is beneficial for the growth of holm oaks and truffles (Büntgen et al., 2015) and, given the global water limitations in view of climate change, drip irrigation has been proposed as a water-efficient system for black truffle cultivation (Magarzo et al., 2023). To enhance water infiltration and truffle production, soil tillage is also applied (Reyna & Garcia-Barreda, 2014; Coleman et al., 2024). Finally, other agricultural techniques often applied in truffle orchards are the host tree pruning and the creation of truffle traps by introducing an organic substrate with supplementary inoculum in the soil (Sourzat, 2020).

Co-cultivation of black truffle and compatible crops

Truffle-growing, either considered as leisure or profitable activity, can barely be the main agricultural activity for interested farmers, given the extended truffle life cycle during the pre-productive period and the unpredictable yields afterwards (Therville et al., 2013). In view of that, a land use- and cost-effective option is the simultaneous cultivation of black truffle and other compatible valued crops, such as aromatic plants, in the same field, at least for the first years since the truffle orchard installation. Apart from diversifying the crop production, and thus the economic profit, in time and space, mixed cropping provides a wide range of benefits in comparison with monocultures, including enhanced biodiversity, pollination, and soil improvement (Burgess et al., 2022). However, the limiting factor in intercropping with black truffle is the brûlé effect, as previously mentioned, that

hinders the growth of nearby plants. Hence, intercropping could be feasible if only compatible crops with black truffle were identified and selected.

Black truffle ecology

Forests where the presence of black truffle naturally emerges are the most representative study areas to better understanding the environmental factors that favour the fungus´ presence. At the same time, productive black truffle plantations are indicative of the human intervention success in somewhat controlling the environmental conditions that favour the fungus´ production. Therefore, both types of black truffle producing systems can be informative about the ecology of the fungus.

Although the focus of interest, either for truffle farmers or researchers, is usually the production of black truffle ascomata, these represent just a part of the fungus' extended life cycle during the year, once the productive period has started. On the other hand, fungal mycelium is present in the soil at a very early stage, since the root-tip's colonisation of the host plant has been established, and its biomass is closely related to the mycorrhizal biomass (Queralt et al., 2017). Thus, the study of mycelium interactions with environmental factors, both abiotic and biotic, could provide some valuable insights for its ecology. Furthermore, tracking inter-seasonal changes in soil mycelium biomass may help to understand the dynamics of fungus´ life cycle in relation with the surrounding environment, facilitating the optimisation of management of truffle-producing systems.

Abiotic environment

Before the planning of a new truffle orchard, the suitability of the area for truffle cultivation is usually evaluated taking into consideration the climate and soil properties (Chevalier & Sourzat, 2012). In fact, climatic conditions are considered critical drivers of truffle production (Garcia-Barreda et al., 2020). Black truffle ascomata are very sensitive to annual climatic conditions due to their long-life cycle, and this is why their natural distribution is limited by summer drought and winter frost (Le Tacon, 2016). It has been suggested that warm spring and wet summer may enhance both the growth of the host tree and the truffle mycelium (Büntgen et al., 2015).

In terms of soil properties, although truffles are able to adapt to numerous soil types, it is well known their preference for alkaline, calcareous soils with structure enabling good aeration and water circulation (Chevalier & Sourzat, 2012; Alonso Ponce et al., 2014; Jaillard et al., 2014).

Previous studies have shown some evidence of the effect of climatic and soil variables on black truffle ascomata at local scale (Castrignanò et al., 2000; García-Montero et al., 2008). Nevertheless, studies of black truffle patterns in relation to the environmental factors at a more extensive spatial

scale, considering distinct abiotic environments and land uses, are scarce but necessary to better understanding the ecology of this fungus.

Biotic environment

Even though black truffle, *T. melanosporum* is, theoretically, the dominant fungal species within the brûlé, much attention has been drawn to the co-existing microbial communities to gain insights into the biotic interactions of black truffle. It is already known that the overall soil fungal diversity is decreased inside the brûlé in comparison with the area outside (Napoli et al., 2010). On the other hand, ECM species may antagonistically interact with the black truffle for host plant and/or niche colonisation (Belfiori et al., 2012). More recently, the abundance of *T. melanosporum* has been found to be negatively correlated with that of arbuscular mycorrhizal fungi and other non-biotrophic fungal guilds (Oliach et al., 2022). In comparison with forests, this competitive effect over the co-occurring fungi may be enhanced in truffle plantations, where the already colonised seedlings are established, and *T. melanosporum* has priority in dominating the space. Indeed, the priority effect has been previously found to affect the microbial composition (Kennedy et al., 2009; Debray et al., 2022). Deciphering the biotic interactions of black truffle with the rest of the fungal community may give valuable information that could inform management strategies to improve black truffle cultivation (Garcia-Barreda et al., 2023).

Black truffle role in the ecosystem

In the natural habitat of black truffle, the complex trophic interactions that take place may determine soil microbial biodiversity, and subsequently the biogeochemical cycles (Erktan et al., 2020), especially the litter decomposition and carbon and nutrient cycling (Hättenschwiler et al., 2005; Grandy et al., 2016). Considering the shift in microbial dynamics inside the brûlé due to the competitiveness or the priority effect of *T. melanosporum*, the presence and/or abundance of the fungus may have a direct or indirect effect at soil functioning-level. Fungi are important decomposers of plant litter and participate in the soil organic matter (SOM) formation through the release of extracellular enzymes that convert litter macromolecules into soluble compounds, which can be used as source of energy and nutrients for plants and microorganisms (Chapin et al., 2011; Baldrian, 2014). In fact, truffles, by forming ectomycorrhizas, participate actively in the biogeochemical cycling and, therefore, play a key role in soil functioning of forests ecosystems and in the maintenance of the food webs (Bonito & Smith, 2016). However, up until now, there are no studies to explore the role of truffles in biogeochemical soil cycles.

Ecosystem services derived from truffle production systems

Ecosystem services (ES) are defined as "*the benefits people obtain from ecosystems*" (Millennium Ecosystem Assessment, 2005) and this concept has been widely accepted as a useful tool for decision-makers involved in environmental management (Przewoźna et al., 2022). ES can contribute on human well-being by providing material and energy needs, by regulating and maintaining the environment, or through cultural characteristics associated with ecosystems that have an impact on physical and mental states of people (Haines-Yong & Potschin, 2018). Although it´s hard to set a value on every way that ES contribute in peoples´ lives, according to the European Investment Bank, the combined benefits of the world's ecosystems contribute an estimated $125 trillion to $140 trillion a year to the economy (https://www.eib.org/en/index).

Truffle producing ecosystems are multifunctional agroforestry or wild forest systems providing an array of ES beyond truffles. Indeed, as previously mentioned, truffle landscapes potentially participate in carbon and nutrient cycling as well as in other ecosystem processes, such as biodiversity conservation or landscape heterogeneity, and they are linked with intangible cultural benefits (Theirville, 2013). However, the relationship between edible mushrooms, like truffles, and ES has been scarcely studied (Nocentini et al., 2022).

Understanding how the local stakeholders involved in the management of truffle systems perceive and value the ES derived by these ecosystems can provide valuable insights for their effective sustainable management (Cabral et al., 2021). With the ultimate purpose to enhance truffle ecosystems conservation, it is considered necessary to include frameworks in land-use policies that integrate ecosystem services (Ruckelshaus et al., 2020). Under the Horizon Europe program (https://research-and-innovation.ec.europa.eu/), the European Commission funds research and innovation that protects biodiversity and preserves ecosystems and their services through improved knowledge and innovation.

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Objectives

The main objective of the thesis is to study the abiotic and biotic interactions of *T. melanosporum* and their linkages with soil functioning in truffle-producing systems, as well as to evaluate the perception and importance that key stakeholders attach to the associated ecosystem services.

The five chapters of this thesis aim to attain the following specific objectives:

1) To identify general variability patterns of black truffle mycelium in relation to soil physicochemical characteristics, seasonality and climate in both productive plantations and truffle-producing forests. For this, the response of black truffle mycelium biomass to a range of environmental variables, in both wild and managed production systems, was analysed across the westernmost native distribution area of *T. melanosporum*.

2) To disentangle the role of *T. melanosporum*, as well as other biotic and abiotic factors, in soil carbon and nutrient cycling. For this, a set of soil enzymatic activities (i.e., as a proxy of soil functioning) involved in carbon, nitrogen and phosphorous cycles were analysed in relation to the truffle abundance and soil properties within the black truffle brûlés of wild and managed black truffle producing systems.

3) To study the soil fungal community composition and fungal co-occurring network structure in black truffle producing systems. For this, soil fungal taxonomic diversity and ecological guilds were determined, and structural properties of the co-occurring fungal network were studied, as well as the taxa associated with *T. melanosporum* and its potential role as a key species in the brûlé.

4) To evaluate the interactions between aromatic plants, truffle hosts and mycorrhizal fungi under an intercropping design. For this, a mesocosm experiment was set up to evaluate the interactions between truffle-inoculated holm oak seedlings and three species of medicinal and aromatic plants, namely lavender, thyme, and sage, either non-inoculated or inoculated with native arbuscular mycorrhizal fungi.

5) To assess the social perception of local stakeholders for the ecosystem services associated to truffle culture in Spain. For this, stakeholders' perception over seven selected ecosystem services was analysed, the main barriers for truffle cultivation and the key reasons that would motivate the implementation of intercropping systems in truffle plantations were assessed.

Chapter 1

Modelling environmental drivers of *Tuber melanosporum* extraradical mycelium in productive holm oak plantations and forests

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Abstract

The black truffle (*Tuber melanosporum* Vittad.) is a highly appreciated edible ectomycorrhizal fungus. It grows belowground and the mycelium and sporocarp production greatly depends on the abiotic environmental conditions. Although there is some evidence about the variables influencing the truffle mycelium at local scale, there is still a lack of knowledge on the soil and climatic patterns driving mycelium growth at broader scales. We aimed to decipher the potential environmental drivers of *T. melanosporum* mycelium across its westernmost natural distribution area. A paired-design experiment with truffle productive plantations and forests was set up across 10 sites. Mycelium biomass was qPCR-quantified, physicochemical soil analyses were done, and climatic data were collected to perform generalised additive modelling. Mycelium dynamics was driven by a combination of soil and climatic variables that accounted for 65.7 % of mycelium variance in plantations and 53.4 % in forests. Longitude, $CaCO_3$, Na and Zn were related, either positively or negatively, with soil mycelium distribution in forests, while elevation, organic matter, P, Mg and B, were the main potential drivers of truffle mycelium biomass in plantations. These results strengthen our knowledge on black truffle ecology and can be applied to design management strategies for optimisation of truffle mycelial biomass and sporocarp production.

Keywords: *Tuber melanosporum*; generalised additive models; black truffle ecology; truffle management; truffle mycelium dynamics

Highlights

- Black truffle mycelium dynamics was modelled in a range of forests and plantations.
- Soil mycelium density was similar between forests and plantations and sampling season.
- Mycelium variability was driven by a combination of edaphic-climatic factors.
- Distinctive drivers of soil truffle mycelium were found in forests and plantation.

1. Introduction

Among the edible fungi trade, truffles exemplify a multimillion business around the world (Oliach et al., 2021), and their cultivation represents a particularly important economic resource in marginal areas (Donnini et al., 2013). Notably, truffle species such as *Tuber melanosporum* Vittad., *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. contribute to agroforestry ecosystems, where inoculated seedlings have been successfully managed to produce truffle sporocarps both within and out of the respective natural distribution ranges of the associated truffle species (Donnini et al., 2013; Reyna & Garcia-Barreda, 2014; Chen et al., 2016). Because of its high profitability, truffle production has promoted the use of agricultural land in low-productive areas through private forestation, as well as important associated ecosystems services such as carbon fixation and soil quality improvement (Leonardi et al., 2021).

The life cycle of black truffle initiates in late winter or early spring, when the released spores germinate and recent hyphae establish ectomycorrhizal symbiosis with the roots of a range of potential host plants, including the representative Mediterranean holm oak tree species (*Quercus ilex* L.). Subsequently, the primordia of truffle ascocarps are formed in May or June, and they mature from November to March of the following year (Payen et al., 2014). Throughout this extended life cycle, soil properties and climatic conditions are considered to be critical for the development of black truffle sporocarps (Jaillard et al., 2016; Le Tacon et al., 2016; Garcia-Barreda et al., 2019). Castrignanò et al. (2000) showed a positive relationship between the growth and production of *T. melanosporum* with soil aggregates and Fe and Mn contents in the brûlé (the area around the tree with little or no vegetation due to the allelopathic effect of truffle mycelium), in a plantation in central Italy. Other soil characteristics such as active carbonate and exchangeable Ca^{2+} have also been found to contribute to truffle sporocarp production and mycelium development in a naturally black truffle-producing forest in central Spain (García-Montero et al., 2007, 2008). Although these studies have provided insights into how soil variables affect the fructification and brûlé size of black truffle at the plot scale, a broader scale view including different land uses (i.e., forest and plantations) is necessary to drawing generalisable conclusions beyond local contexts.

Climatic conditions also play a fundamental role in the persistence and proliferation of ectomycorrhizal fungi, both epigeous (Egli, 2011; Martínez-Peña et al., 2012; De la Varga et al., 2013) and hypogeous like truffles (Garcia-Barreda et al., 2019). Pacioni et al. (2014) associated the increased soil metabolic activity (i.e., CO_2 emissions) targeted by variations in soil moisture and temperature in spring and summer with an intense mycelial growth and fruiting initiation of *T. melanosporum*. Numerous studies at medium-large time scales (Queralt et al., 2017; Baragatti et al., 2019; Büntgen et

al., 2019; Garcia-Barreda et al., 2019) support the idea that truffle production is mainly affected by summer temperature and precipitation. In this line, Le Tacon et al*.,* (2014) showed that water balance in late spring and summer, along with the number of extremely cold days in winter, were the main predictors of annual fluctuations in black truffle production during a 25-yr period in France. In fact, truffle sporocarps develop during winter, and their small and fragile nature makes them susceptible to growth constrains caused by former late-spring-summer water deficit (Baragatti et al., 2019).

A validated method to track the persistence of a given fungus throughout its biological cycle, besides sporocarp´ production, is to detect its extraradical mycelium in the soil using specific DNA probes (Parladé et al., 2016). Tracking inter-seasonal changes in soil mycelium biomass may help to understand the complex interactions between truffle´ life cycle and the surrounding environment, facilitating the optimisation of management of truffle-producing systems. To the best of our knowledge, such a study investigating the relationship between black truffle mycelial biomass and soil properties and/or recent climatic data in orchards and forests at a large spatial scale, has never been conducted before.

Given the tight relationships between the truffle life cycle and the variations in the abiotic environment, firstly, we hypothesised that (i) the truffle mycelium biomass would be dependent on the type of productive system (forests *vs.* plantations) and the season, and that these patterns would be consistent at the regional scale. We expected an increase in truffle mycelium biomass in soils of truffle plantations because of their managed-controlled environmental conditions compared with those in wild forests. Additionally, given the heterothallic nature of truffle mycelium (Riccioni et al., 2008) and the need for compatible hyphae matching for sporocarps formation, greater mycelial biomass was expected in spring than autumn, independently of the production system. Secondly, we hypothesised that (ii) truffle mycelium biomass would respond to different environmental abiotic variables in wild-productive forests and plantations. Given that irrigation is a common practice in truffle orchards, a greater dependency of truffle mycelium on climatic conditions was envisaged in wild-productive forests, as well as a differential response to soil fertility.

To test these hypotheses, we analysed the response of black truffle mycelium biomass to a range of environmental variables in wild and managed production systems, across the westernmost natural distribution area of *T. melanosporum* in Spain. The main objective of our study was to identify general variability patterns of black truffle mycelium in relation to soil physicochemical characteristics and climate in both productive plantations and truffle-producing forests.

2. Materials and Methods

2.1. Study sites and sampling

Sampling sites were selected covering the geoclimatic diversity of the native distribution area of the black truffle in Northeastern Spain. For each site, a paired-design was set to compare two types of truffle production geographically close to each other: a) planted black truffle orchards and b) nearby wild black truffle woodlands (**Fig. 1**), with a total of 10 sites surveyed. All the sampled plantations are productive holm oak monocultures over 10 years, with standard truffle culture management (i.e., soil tillage, irrigation, pruning). In the forest sites, we selected productive *Q. ilex* trees (according to local collectors) in areas dominated by this tree species with an understory composed of different combinations of *Quercus faginea* Lam., *Juniperus* spp., *Crataegus monogyna* Jacq., *Thymus* spp., *Cistus albidus* L. and *Rosmarinus officinalis* L. Concerning the landform of the study sites, for the most part they were flat or, in the case of some natural forests, they had a slight local slope.

The whole study area is characterised by a continental, Mediterranean-type climate with seasonal hydroclimatic variations (**Table S1**). Mean annual temperature and precipitation across sites are 12.3 ± 1°C and 538 ± 136 mm, respectively. Mean altitude of sites is 976 ± 253 m (a.s.l.). To account for plot heterogeneity, we selected at least four productive *Q. ilex* trees for sampling in each plot, with a total of 88 holm oaks monitored that had been previously verified as truffle productive by the orchard owners or the truffle hunters. To account for seasonal variability, soil samplings were conducted at two times in each site, in autumn 2019 and in spring 2020.

A metallic probe was used to obtain soil cylinders of 4 cm diameter and 20 cm deep within the brûlé area of each tree. Four samples per tree were collected from a randomly oriented rectangular area within the brûlé (approximately at the middle point between the trunk and the limit of the brûlé, or at a 50 cm distance from the tree trunk when the brûlé edge was fuzzy) and then pooled in a unique sample per tree. In compacted and rocky soils, found in some forest areas, we excavated 10 x 10 x 20 cm soil holes with a small shovel to take the sample. All the soil samples were stored in plastic bags at 4°C until lab processing.

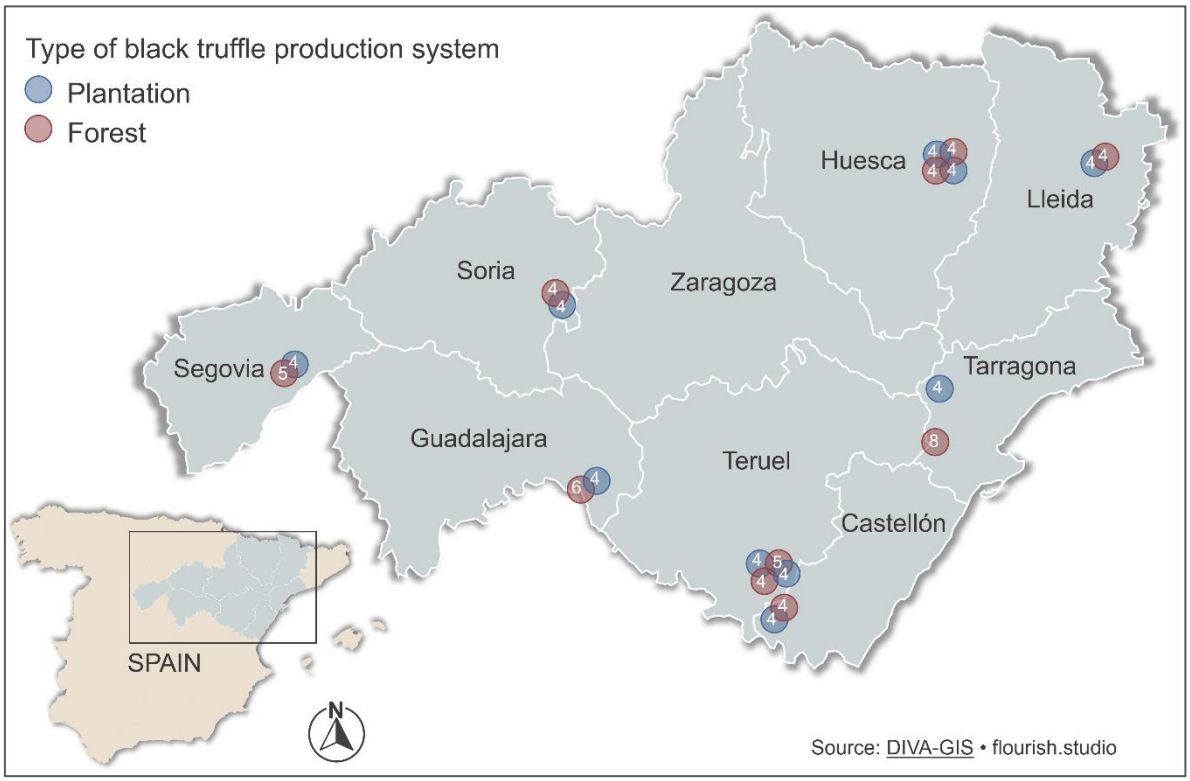


Figure 1 Spatial distribution of the sites selected for this study in nine provinces of Spain, with two different truffle production systems per site: plantation (blue) or forest (red). The number of holm oak trees sampled in each site is noted within the corresponding symbol. Map created with flourish.studio (https://flourish.studio) and adapted by the authors.

2.2. Soil analyses

All the soil samples taken along the two sampling periods were manually homogenised and separated in different aliquots according to the type of analysis. To estimate soil stoniness, a fraction of soil was separated, weighted, and sieved (2 mm) after air-drying. Soil texture (sand, clay and silt %) was determined by the Bouyoucos method (Bouyoucos, 1962). The gravimetric soil moisture (GSM) of soil samples was determined by drying at 105°C for 48 h. Sieved air-dried soils were measured for pH (2 g of soil in 10 ml of H_2O, 1:5, w:v), electrical conductivity (EC) (1:5, w:v in H_2O), organic matter (OM) (Walkley & Black, 1934) and total N (Dumas method; FAO, 2021). Extractable P was determined by the Olsen method, after extraction in 0.5 N sodium bicarbonate solution. Extractable potassium (K), calcium (Ca), sodium (Na) and magnesium (Mg) were determined after ammonium acetate (1 M, pH 7) digestion, and extractable iron (Fe), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni) and boron (B) were determined after DTPA (0.005 M, pH 7.3) digestion. All elements were measured by inductively coupled plasma spectrometry (ICP, Optima 4300 DV, PerkinElmer Inc., Massachusetts, USA). The calcium carbonate ($CaCO_3$) of soils was estimated by gasometric determination of CO_2 with a Chittick apparatus. The active carbonate content was determined by extraction in ammonium oxalate and subsequent potassium permanganate reaction (NF X31-106, 1982). Soil variables were measured

for all samples except texture variables and the content of P and calcium carbonate that were just determined in autumn 2019.

2.3. Climatic data

Climatic data were derived from WorldClim v2.0 (Fick & Hijmans, 2017) with 30 seconds (~1 km^2) spatial resolution. We obtained the data of the average minimum and maximum temperature (°C) of every month and the annual rainfall precipitation (mm) from the historical monthly weather database for the period 2010-2018 (Table **S1**). The Emberger pluvial-thermic index (Emberger, 1932) was additionally calculated for each plot as it summarises adequately the local climatic conditions. This index is used to estimate desertification risks and explain plant species distribution (Vessella et al., 2015; Mitsopoulos & Xanthopoulos, 2016; Jiménez-González et al., 2020). Based on the WorldClim data, the coefficient of Emberger (Q) was calculated as follows: $Q = 100P/(M^2 - m^2)$, (Rubio-Ríos et al., 2022), where P is the annual rainfall, M is the average maximum temperature of the warmest month, and m is the average minimum temperature of the coldest month. Coordinates (latitude, longitude) are omitted from **Table S1** to maintain the privacy of the studied sites, according to the collaborating stakeholders.

2.4. Molecular analyses

The DNA from all the soil samples was extracted from 0.25 g of sieved soil using the DNeasy Power Soil HTP 96 kit (Qiagen, Hilden, Germany), and its concentration was determined by a NanoDrop™ 3300 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). Extracted DNA was stored at -20°C until further analysis.

Mycelium biomass of *T. melanosporum* in each soil sample was quantified by real-time Taqman® PCR (qPCR) with the StepOnePlus™ Real-Time PCR System (Applied Biosystems™ by Thermo Fisher Scientific, Wilmington, USA). The method to create the standard curve for absolute quantification was adapted from Parladé et al. (2007), using the DNA from *T. melanosporum* mycelium instead of sporocarp. For this purpose, we prepared mycelium of *T. melanosporum* in Petri dishes of BAF medium (DSMZ, GmbH, Germany) covered with a cellophane sheet (**Fig. S1**). The plates were inoculated with an active-growing colony of *T. melanosporum* and incubated at 25°C for two months until an active growth of the mycelium was obtained. Then, the mycelium was removed from the cellophane using a scalpel, pooled into an Eppendorf tube, and dried at 50°C for 1 h in a heating chamber (ED-S 115, ©BINDER GmbH, USA). We extracted the DNA from a known weight (7 mg) of dried mycelium using the Soil DNA Isolation Plus Kit (Norgen Biotek Corp., Canada). The standard curve was created based on the C_T values of a ten-fold dilution series of the mycelium DNA, from 10^0 to 10^{-4}. The reaction mix

and cycling conditions of qPCR were as described in Parladé et al., (2013). Three technical replicates per soil sample were included in the reaction plates. The C_T values of soil samples were then interpolated into the standard curve to calculate the mycelium quantity of *T. melanosporum* (Parladé et al., 2007; De la Varga et al., 2013), expressed in µg of mycelium per g of soil. To perform and interpret the qPCR assays, the MIQE (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines, proposed by Bustin et al. (2009) were considered.

2.5. Statistical analyses

Truffle mycelium biomass and environmental variables were checked for normality by the Shapiro-Wilk test and the error distribution of data was checked and considered for model building.

2.5.1. Effect of the type of production and the season on truffle mycelium biomass

To test the effect of the production type (forest *vs*. plantation), the season of sampling (autumn *vs*. spring) and their interaction on truffle mycelium variance (hypothesis 1), considering spatial-temporal random effects, we first fitted linear mixed models to the paired-design dataset (*N* = 176). However, the non-normal distribution of the residuals indicated the use of generalised linear mixed models (GLMMs). Furthermore, as an extension of GLMMs, generalised additive mixed models (GAMMs) allowed to fit both linear (similarly to GLMM) and non-linear terms, without assuming a priori a specific type of relationship between the dependent variable and the predictors. In this step, a null generalised additive mixed model (hereafter 'null GAM') was set to describe the effect of the linear fixed terms 'type' and 'season' on truffle mycelium variance. The R syntax for the null GAM was set as:

$$gam(mycelium \sim type * season + s(site, bs = "re") + s(site, by = season, bs = "re"), family = Gamma(link = "log"))$$

where 'mycelium' is the response variable, 'type' of production system and 'season' are the fix factors included as linear predictors, 'site' and 'site' nested in 'season' (accounting for spatial variation and repeated measures, respectively) are the random effects ('re') included as non-linear predictors by applying the spline smooth function *s*() to fit non-parametric regression, and 'Gamma' is the error distribution function. GAMs were run with the *gam* function of the 'mgcv' R package (Wood, 2008), and the distribution of residuals was checked for normality and homogeneity by the *gam.check* function before model validation.

2.5.2. Response of truffle mycelium to abiotic environmental factors

Next, we analysed the environmental factors affecting the truffle mycelium biomass distribution in productive plantations and forests (hypothesis 2). In a first step, the collinearity among all environmental variables (26 in total) was analysed by Pearson correlation, using the *rcorr* function in the 'Hmisc' R package (Harrell, 2019) and plotted by the *corrplot.mixed* function of the 'corrplot' R package (Wei & Simko, 2021). In a second step, variables were grouped as 'geoclimatic factors' (G), 'soil fertility' (F) and 'other edaphic properties' (E) (Table **1**) to examine whether a specific group of variables or any combination of them would show high relative contribution in explaining truffle mycelium variation.

Table 1 Groups of explanatory variables used in this study: geoclimatic, soil fertility and other edaphic properties.

Geoclimatic (G)		**Fertility (F)**		**Other edaphic properties (E)**	
Emberger coefficient	(Q)	N	(%)	Sand	(%)
Elevation	(m.a.s.)	P	(mg kg^{-1})	Clay	(%)
Latitude	(°)	K	(mg kg^{-1})	Silt	(%)
Longitude	(°)	Ca	(mg kg^{-1})	Stoniness	(%)
		Na	(mg kg^{-1})	pH	
		Mg	(mg kg^{-1})	Gravimetric soil moisture	(%)
		Fe	(mg kg^{-1})	Electric conductivity	(µs cm^{-1})
		Zn	(mg kg^{-1})	Organic matter	(%)
		Mn	(mg kg^{-1})	Active carbonate	(%)
		Cu	(mg kg^{-1})	Calcium carbonate	(%)
		Ni	(mg kg^{-1})		
		B	(mg kg^{-1})		

Finally, we fitted models with all possible variable combinations, independently of their previous assignation in **Table 1**. For this, a 'full subsets generalised additive modelling (GAM)' approach was applied by using the FSSgam R package (Fisher et al., 2018). This procedure consists of fitting models of all possible variable combinations (subsets) with up to a maximum of three predictor variables per model (to ensure that the models will remain ecologically interpretable), comparing all resulting GAMs and identifying the best-fitted ones through stepwise selection based on the Akaike's information criterion (AIC) and parsimony principle. The continuous variables were specified as smooth terms and the degrees of freedom (*df*) were restricted to 4 for each smoother ($k = 4$) to avoid models over fitting, as recommended by Fisher et al. (2018). The factors 'type' and ´season´ were included as linear predictors and the random effects were added to all model construction, as previously described in null GAM. All models were fitted to a Gamma distribution and log-link function. To tackle with the high collinearity among continuous variables, the Pearson's correlation cut-off in the FSSgam package was limited to 0.28.

This analytical procedure was applied to the complete dataset (*N* = 176), and separately to the truffle plantations (*N* = 80) or forests (*N* = 96) datasets (in these cases the term 'type' was excluded from models). The explanatory variables were sequentially added in models to get seven model's clusters: 1) using only geoclimatic characteristics (model 'G'), 2) only soil fertility (model 'F'), 3), only other edaphic properties (model 'E'), 4) both geoclimatic factors and soil fertility (model 'GF'), 5) both geoclimatic factors and edaphic properties (model 'GE'), 6) both other edaphic properties and soil fertility (model 'EF') and 7) altogether geoclimatic factors, other edaphic properties and soil fertility (model 'GEF') (Table **S4**). After obtaining all possible models, the top-ranked ones of each cluster were selected among those that showed less than two AICc units of difference respect to the most parsimonious model (lowest AICc) (i.e., ΔAICc < 2) (Burnham & Anderson, 2004). The normal distribution of residuals and the absence of autocorrelation effects were checked for each model.

Once the top-ranked models were selected, the relative contribution of each group of variables in explaining truffle mycelium variation was determined based on the GAMs deviances explained (DEs) (Table **S4**), according to Zhou et al. (2021). For this, the unique contribution (the deviance exclusively explained by a single group of variables, i.e., model scenarios 'G', 'E' and 'F') and the common contribution (the deviance explained by multiple groups of variables, i.e., model scenarios 'GE', 'GF', 'EF' and 'GEF') of variables, as well as their respective differences were quantified as indicated in Table **S4**. Finally, for the model scenario with the highest relative contribution on truffle mycelium variance, the relative effect of each individual explanatory variable was calculated. For that, the importance of each explanatory variable across all candidate models was estimated by the summed AICc weights (second order information criterion, taking into account the sample size) of all the models where the explanatory variable appeared, ranking as more important those predictors with higher summed values. The relationships among the most relevant individual explanatory environmental variables with the truffle mycelium biomass were plotted with the *plot smooth* function of the 'itsadug' R package (van Rij et al., 2022).

All statistical analyses of this study were performed with the R software v.4.2.1 (R Core Team, 2022).

3. Results

3.1. Effect of the production type and the season on soil truffle mycelium biomass

Overall, forests had higher average mycelium biomass of *T. melanosporum* than plantations in both seasons, and in autumn the average mycelium showed higher values than in spring in both production types (**Fig. 2**). However, when the site was taken into account in the analysis, the null model (GAM) showed that just the season and the random effects (i.e., spatial-temporal factors), but not the type of production, were significant predictors of truffle mycelium (42.2 % deviance explained; AIC = 1676.73) (**Table S2**).

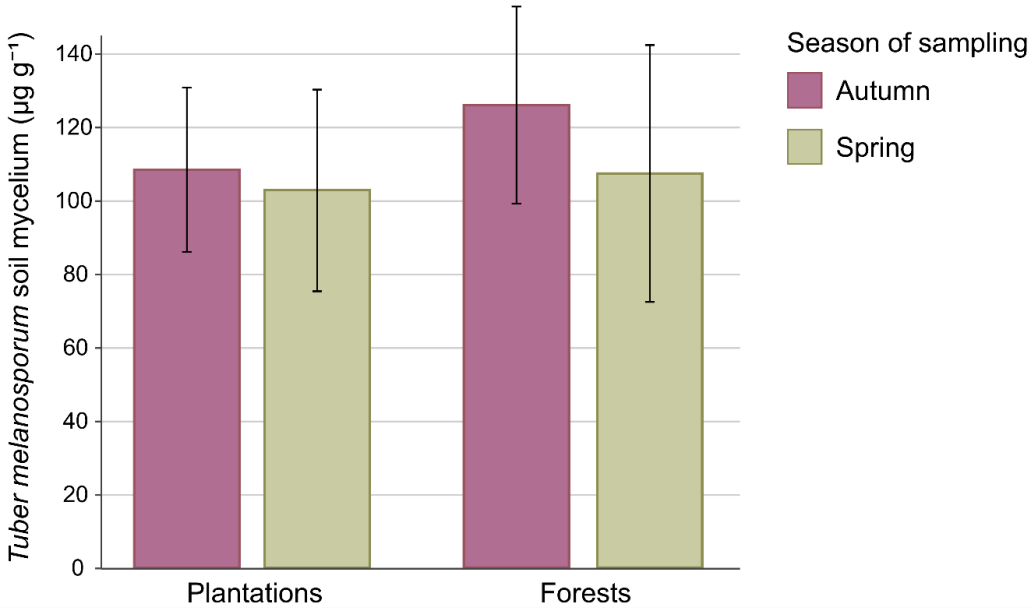


Figure 2 Mean mycelium biomass of *Tuber melanosporum* in soils of two black truffle production systems: plantations and forests, at two seasons: autumn and spring. Error bars denote 1SE.

3.2. Relative contribution of edaphoclimatic factors to the truffle mycelium biomass variance

Mean values and variation of the climatic and soil variables analysed in this study are shown in **Table S3**. These environmental variables showed high collinearity and, except for soil sand and pH, most of the correlations observed were positive (**Fig. S2**).

When the entire dataset was modelled as a function of general groups of explanatory variables (geoclimatic factors G, soil fertility F, and other edaphic properties E), the best-fitted models showed values of truffle mycelium deviance explained between 41.1 – 45 % (**Table S4**). Compared with the whole dataset, the separate analysis of truffle plantations and forests datasets gave better yields for all model parameters e.g., mycelium deviance explained: 67.5 – 70.5 % for plantations and 43.6 – 57.4

% for forests (**Table S4**). Within each dataset, the seven best-fitted models gave quite similar results. However, compared with forests, all the best-fitted models in truffle plantations had the highest deviance explained and the lowest AIC, indicating that the environmental variables tested were better predictors of the mycelium biomass variability in plantations than forests.

When the relative contribution of each group of environmental variables was calculated, it was showed that none group i.e. geoclimatic G, other edaphic properties E, soil fertility F, had a predominant separate weight explaining truffle mycelium variation when they were considered by pairs or alone, but they did have when considered together (rates of mycelium variance: 42.6 % total dataset, 65.7 % plantations, 53.4 % forests) (**Fig. 3**; **Table S4**).

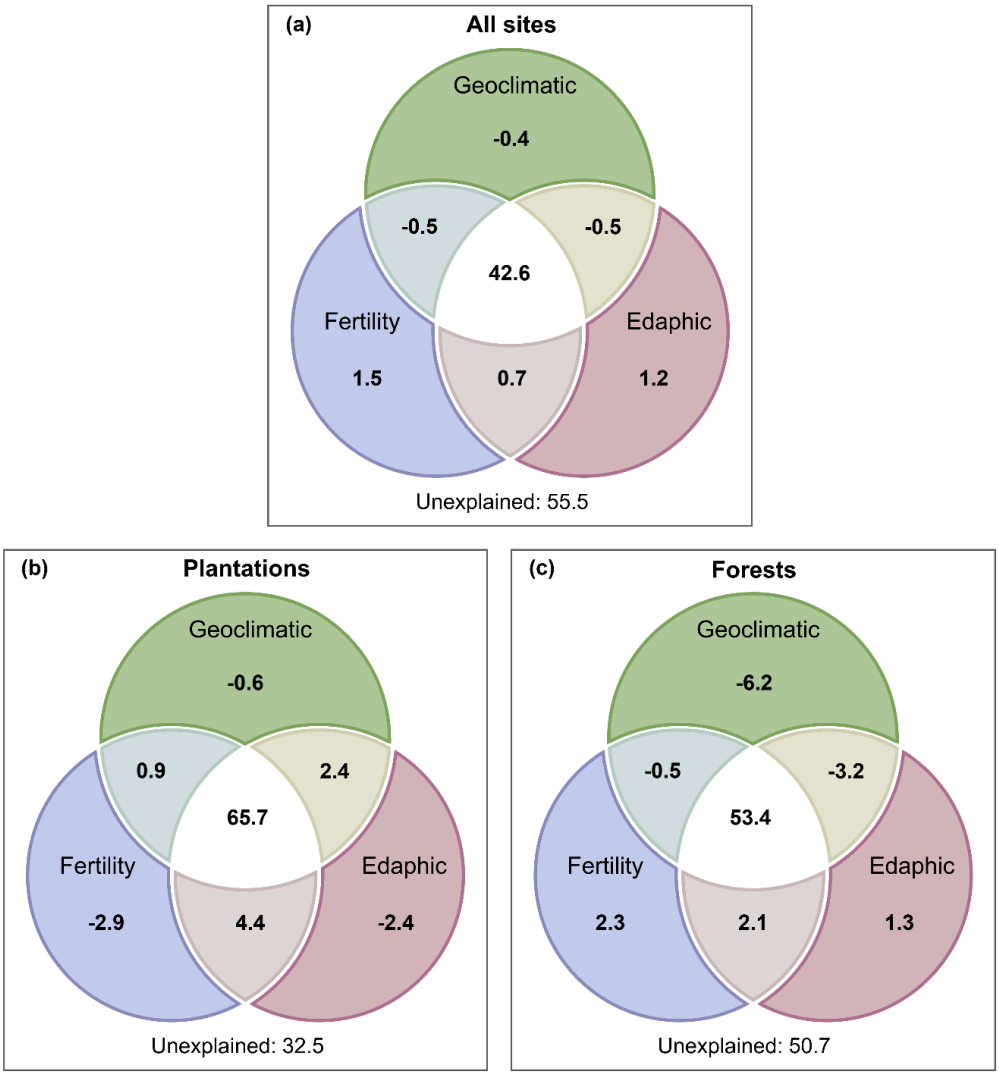


Figure 3 The unique and common contributions (%) of three environmental scenarios: geoclimatic factors (G), soil fertility (F), other edaphic properties (E) and their combinations (GE, GF, EF, GEF) in mycelium biomass variance of *Tuber melanosporum* across **(a)** all sampled sites, **(b)** truffle plantations and (c) forests. The relative contribution of each scenario was quantified by the deviances explained (DE) of the best-fitted generalised additive models (GAMs) calculated as indicated in Table **S4**. Negative rates resulted when the combination of a single group or two groups of variables (GE, GF, or EF) explained more deviance than the combination of the three groups of variables (GEF) (see Table **S4**).

3.3. Effect of individual variables in truffle mycelium biomass variance

Since the combination of geoclimatic factors G, soil fertility F, and other edaphic properties E had the highest explicative potential of *T. melanosporum* mycelium variation, more attention was drawn to the best-fitted GAMs considering all variables (i.e., independently of their previous grouping). The full subsets analysis considering all possible variable combinations (subsets) with up to a maximum of three predictor variables yielded a total of 761 and 848 GAMs in the datasets of plantations and forests, respectively. The top-ranked models of this 'full subsets analysis' for plantations and forests reported only two with ΔAICc < 2 (**Table 2**) for each type of production system. When the importance of each predictor within the top-ranked models was estimated (i.e., by the summed AICc weights in **Table 2**), in general, scores were lower in plantations than in forests, and the variables that were respectively most influencing in each truffle-producing system were different (**Fig. 4**). The best model (ΔAICc = 0) for predicting truffle mycelium biomass in plantations (**Table 2**) showed significant contributions of elevation, organic matter and magnesium (**Fig. 4a**), whereas for forests it included longitude, calcium carbonate and sodium (**Fig. 4b**).

Table 2 Summary results from the 'full subsets analysis' showing generalised additive models (GAMs) with ΔAICc < 2 respect to the most parsimonious model (that with lowest AICc) for black truffle mycelium prediction, in truffle plantations and forests. AIC corrected for small sample size (AICc), difference from lowest reported AICc (ΔAICc), AICc model weights (wi.AICc), deviance explained (DE) and estimated degrees of freedom (edf) of the respective GAMs are reported.

Dataset	Model	AICc	ΔAICc	wi.AICc	DE	edf
Plantations	Elevation + organic matter + Mg	788.45	0	0.12	0.68	16.38
	Elevation + P + B	789.83	1.37	0.06	0.70	17.08
Forests	Longitude + CO_3Ca + Na	843.18	0	0.26	0.49	19.04
	Longitude + Na + Zn	844.41	1.23	0.14	0.48	18.18

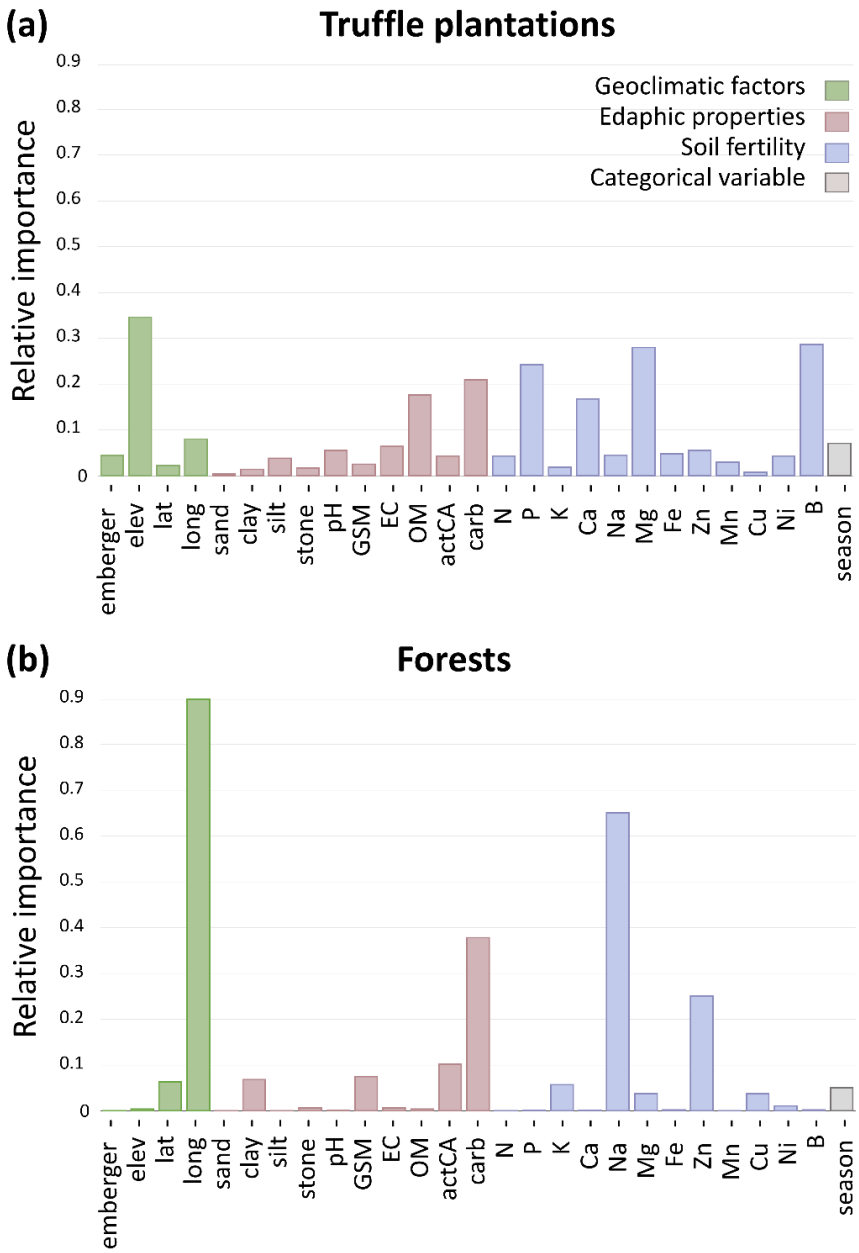


Figure 4 Variable importance scores from a full subsets analyses exploring the effect of geoclimatic factors (green), edaphic properties (pink) and soil fertility (purple) on *Tuber melanosporum* mycelium biomass in **(a)** truffle plantations and **(b)** forests. Variable importance was calculated by the summed AICc weights of all the models that each predictor appeared through the model construction process. Emberger = Q index = 100*P* (annual rainfall)/M^2 (average max. temperature of the warmest month) – m^2 (average min. temperature of the coldest month), lat = latitude, long = longitude, elev = elevation, GSM = gravimetric soil moisture, EC = electric conductivity, OM = organic matter, actCA = active carbonate, carb = calcium carbonate.

In plantations, truffle mycelium biomass slightly decreased between 400-800 m, but increased at higher elevations (**Fig. 5a**). Soil organic matter and magnesium content had a positive linear relationship with truffle mycelium biomass (**Fig. 5b,c**). Regarding the forests, truffle mycelium first increased at approximately -4° to 0° longitude and then decreased with increasing values (i.e., in

eastern sites) (**Fig. 5f**). Mycelium biomass showed a nonlinear relationship with calcium carbonate content in soil, with a negative trend at rates up to 20% and above 60% and a positive trend at intermediate rates (**Fig. 5g**). Truffle mycelium biomass in forests showed a negative relationship with the sodium content in soil (**Fig. 5h**).

As regards the second best-fitted GAMs (plantations: elevation + P + B; forests: longitude + Na + Zn; Table **2**), in truffle plantations, the relationship with phosphorous content was slightly positive at low levels, and then it became negative at rates over 20 mg kg^{-1}, whereas boron levels had a positive influence on mycelium biomass with an asymptotic relationship, with a limit of the positive trend over 0.10 mg kg^{-1} soil (**Fig. 5d,e**). In forests, the truffle mycelium showed a negative relationship with the content of zinc (**Fig. 5i**).

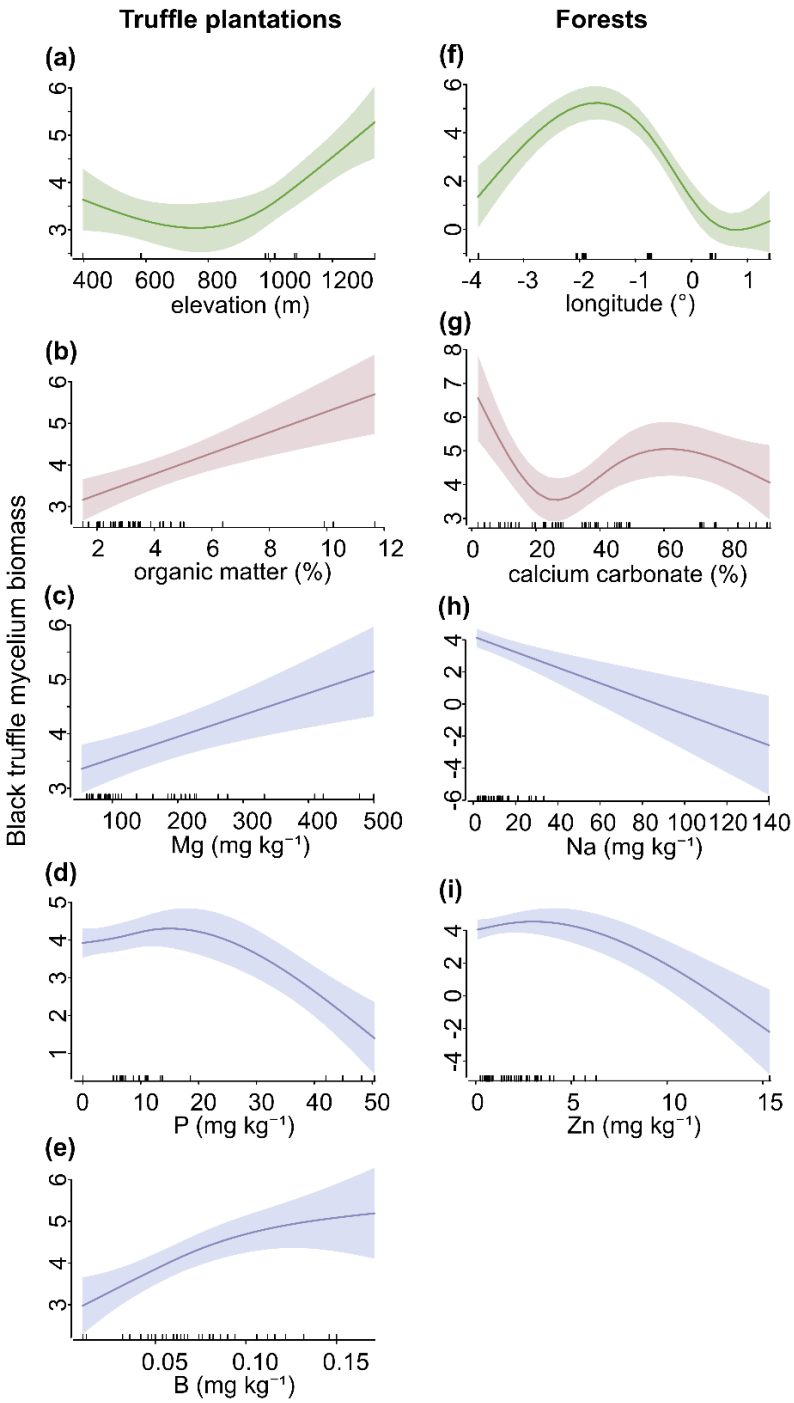


Figure 5 Partial effect of the most important environmental variables explaining black truffle mycelium biomass in plantations (**a**, **b**, **c**, **d**, **e**) and forests (**f**, **g**, **h**, **i**), based on the AIC of the most parsimonious generalised additive models (GAMs) (a, b, c, f, g, h) and the second best-fitting GAMs (d, e, i). The solid line indicates the estimated smoothing curve and the shaded areas indicate the 95% confidence interval of the regression line. Green represents geoclimatic factors (G), pink represents edaphic properties (E) and purple represents soil fertility (F) variables.

4. Discussion

The relative importance of environmental variables as potential drivers of the extraradical mycelium of *T. melanosporum* was assessed along two seasons, in truffle orchards and forests in Spain. Studying truffle mycelium dynamics is relevant since fruiting body production has been positively related to mycelium in local studies involving truffles (Suz et al., 2008; Zampieri et al., 2012; Queralt *et al*., 2017). This relationship has also been observed in the common practice consisting of placing fungal material into small pit dugs under truffle host trees (truffle traps), where the stimulation of the vegetative development of maternal mycelium is achieved and the fruitbody production is favoured (Taschen et al., 2022).

Contrary to hypothesis 1, we found that the amount of truffle mycelium was independent of the type of production, and overall mycelium biomass was higher in autumn than in spring. However, while the sampling season was a significant predictor for mycelium variability with the entire dataset (i.e., null GAM), it was not the case for the datasets of truffle plantations and forests separately. Previous studies have related intra- and inter- annual variability of black truffle production with climatic factors (Büntgen et al., 2015; Garcia-Barreda et al., 2020). Nevertheless, no information is available on the spatial and temporal patterns of soil mycelium of this fungus at a large scale. Castaño et al. (2017) also observed peaks of extraradical mycelium of *Lactarius vinosus* (Quél.) Bataille in spring and autumn in a Mediterranean forest. However, a similar study in a *T. melanosporum* plantation (Queralt et al., 2017) showed a different seasonal variation pattern of mycelium biomass in two consecutive years, and a significant correlation with the accumulated precipitation of the month previous to sampling throughout the year. Thereby, it seems that the mating events of truffle mycelium in spring do not involve an increase of soil mycelium biomass in *T. melanosporum* and that the temporal pattern is rather related to local soil-climatic factors.

Regarding the type of production, it is known that *T. melanosporum* outcompetes other ectomycorrhizal fungi in the brûlés (Napoli et al., 2010). In fact, Oliach et al. (2020) did also find that holm oaks inoculated with *T. melanosporum* and planted in areas surrounded by forests were colonised by other ectomycorrhizal fungi, but even so, the abundance of *T. melanosporum* mycelium was not reduced in the proximity of the forest. Our data also support that ectomycorrhizal competition in productive forests does not affect the soil density of *T. melanosporum* mycelium, as compared to monospecific plantations.

According to our second hypothesis, truffle mycelium biomass did respond to different abiotic variables in wild-productive forests and plantations. Modelling the geographic characteristics, edaphic properties and soil fertility variables as potential drivers of black truffle mycelium biomass showed

that, in every model scenario, the best-fitted model explained 11-25 % more deviance in plantations than in forests. Similarly, the common contribution of the environmental variables to mycelium biomass was 12 % higher in plantations than in forests. This result may be related to the fact that truffle plantations have been subject of long-term cultivation practices, such as tillage, simplifying, this way, the soil environment and decreasing the background noise. Besides the underlying differences in the soil-climatic properties of the two productive systems, these results indicate that other variables (e.g., biological interactions and soil structure) not taken into account in this study are likely to be relevant explaining *T. melanosporum* mycelial variance, particularly in unmanaged forest systems. For example, different biological interactions with the soil microbial community and/or different soil structure that may affect the nutrient availability to soil microorganisms (Erktan et al*.,* 2020) could have accounted for the unexplained part of our models. In addition, the relative importance of variables was clearly different in both production types, as well as the key variables explaining truffle mycelium variation in each system. The differences found could be explained in terms of different soil development and biology of plantations *vs.* forests, and/or previous land use in plantations (Therville et al., 2013; Flores-Rentería et al., 2016).

Among the specific variables identified by the 'full subsets analysis' to explain mycelium variation, the elevation of sites had a significant positive relationship with truffle mycelium in plantations, but not in forests. This could be related to the drastic reduction during Mediterranean summer season, highlighting the importance of soil moisture for black truffle production (Garcia-Barreda et al., 2019). This altitude effect would be less evident under the canopies of the sampled forests with soils richer in organic matter, and with higher water holding capacity (Hudson, 1994).

Truffle mycelium biomass in forests showed a bell curve shaped relationship with longitude, compatible with the agro-climatic zones of black truffle productive forests proposed by Garcia-Barreda et al. (2019) in the Iberian Peninsula. The distribution pattern of fungal mycelium in our samples showed a longitudinal zonation with the highest mycelium abundance in Soria and Guadalajara provinces, corresponding to the western areas of our study.

Regarding soil factors, the mycelium biomass had a positive and linear relationship with soil organic matter in truffle plantations. Different authors have shown the association between soil fungal mycelium and organic matter content and fractions (Soukupová et al., 2008; Hu et al., 2023; Raza et al., 2023). Compared to forests, long-term cultivation of agricultural land can result in decreased content of soil organic carbon (Beheshti et al., 2012). Indeed, lower organic matter content was observed in plantations than forests and this could possibly indicate a higher dependence of truffle mycelium biomass on organic matter quantity and quality, in soils with less availability.

Calcium carbonate was a relatively important variable for *T. melanosporum* mycelium in both forests and plantations, even if in the latter case the variable was not present in the best-fitted models. This agrees with the results of Gryndler et al. (2017) regarding the positive effect of calcium carbonate on *Tuber aestivum* mycelium and with those of García-Montero et al. (2007, 2008) about the contribution of active carbonate and exchangeable Ca^{2+} to sporocarp production and burn size of *T. melanosporum.* Moreover, García-Montero et al. (2024) have recently shown the ongoing formation of active carbonates inside the brûlé, suggesting the active habitat modification and niche construction by the black truffle.

Regarding soil fertility, the magnesium content had a positive linear relationship with mycelium biomass in truffle plantations (generally lower than in forests). Magnesium is an essential element, the deficiency of which affects photosynthesis and carbohydrate partitioning in crops (Farhat et al., 2016; Wang et al., 2020). Soil carbon allocation depends on nutrients such as Mg (Ericsson, 1995) that are closely related to photosynthesis, and any change in C allocation can affect mycelium growth of ectomycorrhizal fungi (Bahr et al., 2013). We suggest that the positive effect of Mg on truffle mycelium observed in our study might be related to the improvement of the photosynthetic activity of the host plant and thus the higher carbon deliver for fungal mycelial growth.

We also found a positive trend of boron on mycelium biomass in plantations. B can affect the soil microbiome (Vera et al., 2021) and play a role in plant cytoskeleton formation (Bassil et al., 2004), which may be also related to the mycorrhizal establishment (Lehto et al., 2010). The availability of B increases in alkaline soils, such as those appropriate for truffle production as in our study. Our plantations were slightly more alkaline than the forest sites, which possibly enhanced B content in soil, favouring also the mycorrhizal association with plants.

Truffle mycelium biomass was negatively related with sodium in forests. Although all the studied sites were within the distribution area of saline and sodic soils in the European Union (Tóth et al., 2008), their Na^{+} values were within the range measured in productive black truffle plantations in the province of Teruel, NE Spain (Alonso Ponce et al., 2014). Surprisingly, this variable did not appear as relatively important to explain the variation of mycelium biomass in plantations, where the mean Na^{+} amount in soil was higher than in forest areas probably because of the secondary soil salinisation occurring in agricultural drylands through water logging or improper irrigation systems (Stavi et al., 2021).

The relationship of phosphorous with truffle mycelium in plantations at low levels was positive, but after a certain threshold, it displayed a negative trend. Zhang et al. (2019) found both positive and negative relationships of exogenous P and N on *Tuber indicum* Cooke & Massee colonisation depending on the application dosage, while it is well known that high P concentrations in soil can inhibit

mycorrhiza establishment and root colonisation (Smith & Read, 2008). Forests sites showed less bioavailable P than plantations, possibly related to indirect negative effects of drought in Mediterranean forests (Sardans & Peñuelas, 2004) compared with well-irrigated plantations. Besides, truffle plantations were probably established in previously cultivated fields where fertilisation may have been applied, increasing the P content in the soil.

Soil Zn was negatively related to mycelium biomass in forests. Zinc is an important micronutrient for plants, whose deficiency in alkaline soils creates hurdles in achieving optimum crop growth (Saboor et al., 2021). Zinc can become an environmental concern at toxicity levels, although our sampled soils are well below the limits considered toxic for plants (Noulas et al., 2018; Kaur & Garg, 2021).

In addition to abiotic environmental factors that interact with black truffle, a recent study has identified some fungal and bacterial species that co-occur with *T. melanosporum* and may drive its dynamics in soils of truffle plantations (Herrero de Aza et al., 2022). Other authors have pointed that the capacity of *T. melanosporum* to expand in the soil and uptake nutrients might be facilitated but also interfered by the surrounding microbial community (Rubini et al*.,* 2014). While in natural forests the *Tuber* spp. colonisation depends on fungal competition, in truffle orchards (i.e., agriculture soils depleted of ectomycorrhizal propagules), the inoculated plants are advantageous for the development of the introduced fungus provided the absence of competition with other ectomycorrhizal species (Leonardi et al., 2021). Čejka et al. (2023) suggested that the identification of common fungal taxa across *Tuber magnatum* Pico sites and their attribution to functional guilds would help to distinguish productive sites from unproductive ones. Further studies on microbial communities that coexist with *T. melanosporum* will help to evaluate the ecology and successful establishment of this fungus in the soil.

5. Conclusions

The dynamics of black truffle mycelium in our study is driven by a combination of climatic and soil factors, rather than by a particular effect of either climatic or soil properties. The combination of the studied environmental factors accounted for almost two thirds of the mycelium variance in plantations and half of the mycelium variance in forests, whereas the groups of variables alone or in dual combinations explained a negligible part (< 5 %) of the mycelium variation. At the regional scale (i.e., independently of the site), the relative contribution of explanatory variables showed that longitude, carbonates, Na and Zn were related, either positively or negatively, to soil mycelium distribution in forests. In plantations, the elevation, content of organic matter, P, Mg and B were related to the regional distribution of soil mycelium. Our results give important clues on black truffle ecology and are highly valuable for applied purposes e.g., the design of tailored fertilisation schedules to

maximise fungal biomass, assuming that this will improve truffle production. Further studies on truffle ecology are needed to fully understand the environmental drivers of black truffle mycelium. In any case, accurate data on truffle production would notably help to design fruiting models to be compared with those developed for soil mycelial biomass in this study.

Author contributions

Design of the research, Javier Parladé and Ana Rincón; field and laboratory tasks, Javier Parladé, Ana Rincón and Vasiliki Barou; data analysis, collection, or interpretation, Javier Parladé, Ana Rincón and Vasiliki Barou; and writing the manuscript, Javier Parladé, Ana Rincón and Vasiliki Barou. All authors contributed to the manuscript edition and approved the final version.

Declaration of Competing interest

The authors have no competing interests.

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Data availability

Data will be made available from the corresponding author upon reasonable request.

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7. Supplementary material

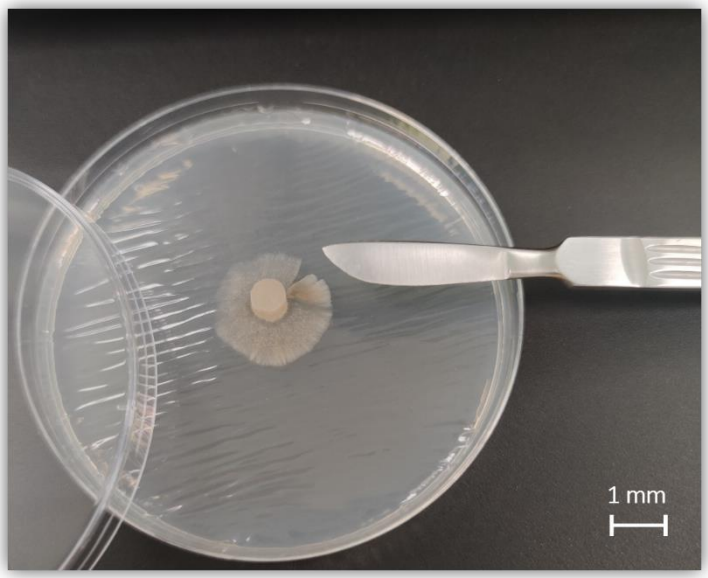


Figure S1 Cultivation of *Tuber melanosporum* mycelium. The fungus was grown in a Petri dish with BAF medium covered with cellophane.

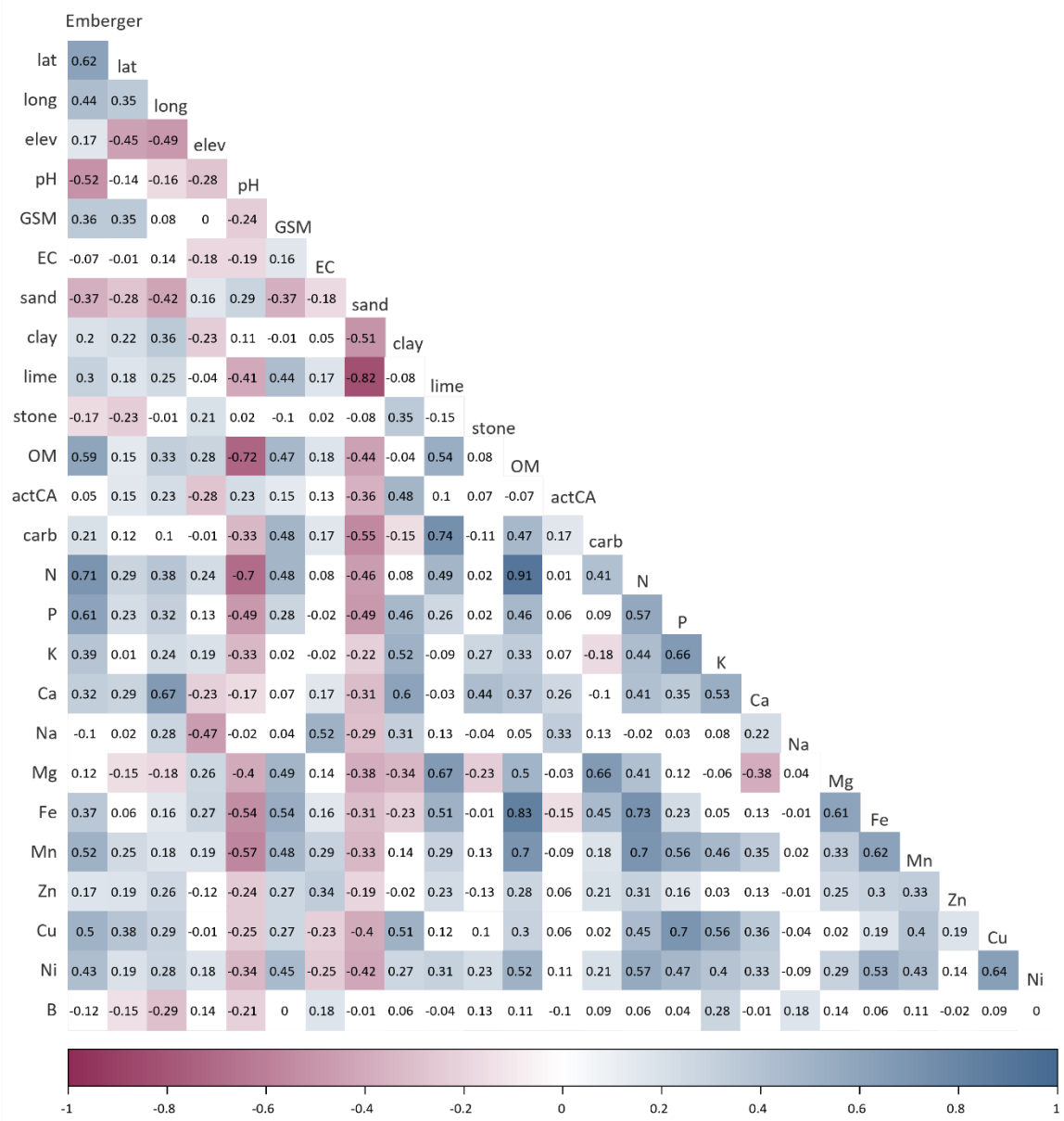


Figure S2 Correlation matrix of geoclimatic factors, edaphic properties and soil fertility variables of soil samples. Data represent samples in truffle producing plantations and forests, collected in autumn 2019 and spring 2020 (*N* = 176). Significant correlation coefficients (Pearson p <0.05) are shown with colour gradient (blue, positive correlation to red, negative correlation) and non-significant coefficients are white. Emberger = emberger index, lat = latitude, long = longitude, elev = elevation, GSM = gravimetric soil moisture, EC = electric conductivity, OM = organic matter, actCA = active carbonate, carb = calcium carbonate.

Table S1 Number of trees selected for sampling and geoclimatic variables of each site. Data were derived from WorldClim database for the period 2010-2018. Precip = Mean annual precipitation, Temper_warm = Mean temperature of the warmest month, Temper_cold = Mean temperature of the coldest month, Temperature = Mean annual temperature.

ID	Site (Province)	Production type	Nº trees	Elevation (m.a.s)	Precip (mm)	Temper_warm (°C)	Temper_cold (°C)	Temperature (°C)
1	Lleida	Plantation	4	1079	829.20	26.23	-1.80	10.43
2	Lleida	Forest	4	1079-1183	829.20	26.23	-1.80	10.43
3	Tarragona	Plantation	4	398	459.30	32.65	2.09	15.46
4	Tarragona	Forest	8	1022-1087	592.17	29.03	-1.14	11.84
5	Teruel_1	Plantation	4	1013	401.90	31.82	-2.37	12.41
6	Teruel_1	Forest	4	988-1118	402.54	31.64	-2.40	12.31
7	Teruel_2	Plantation	4	984-994	405.11	31.50	-2.07	12.47
8	Teruel_2	Forest	5	979-1110	387.70	31.78	-1.93	12.71
9	Castellón	Plantation	4	1015	418.88	31.06	-2.52	11.85
10	Castellón	Forest	4	1024-1041	409.13	31.61	-2.49	12.16
11	Huesca_1	Plantation	4	586	667.96	31.42	0.70	13.70
12	Huesca_1	Forest	4	631-656	667.96	31.42	0.70	13.70
13	Huesca_2	Plantation	4	583	611.63	32.31	1.33	14.50
14	Huesca_2	Forest	4	583	606.43	32.34	1.34	14.52
15	Guadalajara	Plantation	4	1336	584.33	29.49	-3.40	10.45
16	Guadalajara	Forest	6	1221-1372	586.23	29.33	-3.32	10.44
17	Segovia	Plantation	4	1085	478.617	29.12	-1.25	11.55
18	Segovia	Forest	5	1022-1086	453.74	29.24	-1.00	11.77
19	Soria	Plantation	4	1159	473.51	30.09	-1.55	11.63
20	Soria	Forest	4	1133-1147	493.03	29.76	-1.90	11.29

Table S2 Results of the null generalised additive model (null GAM). The results describe the response of *Tuber melanosporum* soil mycelium according to the type of truffle production (plantation vs. forest), the season of sampling (autumn vs. spring), the interaction of type and season and the site of sampling as random effect (ten sites across its natural distribution area in Spain, *N* = 176). edf = degree of freedom, Ref. df = reference degree of freedom.

Linear terms	Estimate	Std Error	t-value	p-value
(Intercept)	4.494	0.264	17.036	0.0000 ***
type	-0.211	0.256	-0.827	0.4094
season	-1.395	0.512	-2.726	0.0071 **
type:season	0.426	0.362	1.179	0.2402
Smooth terms	**edf**	**Ref. df**	**F-value**	**p-value**
s(site)	5.858	9.000	34.081	0.0000 ***
s(site):season_autumn	0.781	9.000	0.275	0.0650
s(site):season_spring	7.836	9.000	73.081	0.0000 ***

**, $P < 0.01$; ***, $P < 0.001$

Table S3 Mean ± standard deviation (SD) values of soil properties of the truffle plantations and forests analysed in this study. GSM = gravimetric soil moisture, EC = electric conductivity, OM = organic matter, actCA = active carbonate, carb = calcium carbonate.

Edaphic variable (units)	Plantations		Forests	
	Mean	SD	Mean	SD
pH	8.25	0.29	8.09	0.25
GSM (%)	11.71	3.28	11.27	5.61
EC (µs cm^{-1})	273.66	126.62	276.34	129.41
sand (%)	46.18	12.54	52.12	13.85
clay (%)	25.74	7.49	16.72	5.16
silt (%)	28.08	8.48	31.16	13.76
stone (%)	36.57	21.32	38.44	18.99
OM (%)	3.70	2.11	5.6	3.17
actCA (%)	6.97	4.27	4.95	3.24
Carb (%)	30.55	19.51	39.2	25.41
N (%)	0.13	0.10	0.18	0.11
P (mg kg^{-1})	8.89	13.59	2.94	3.66
K (mg kg^{-1})	165.13	78.64	126.88	54.31
Ca (mg kg^{-1})	3246.24	1062.77	3175.35	987.72
Na (mg kg^{-1})	17.08	24.39	9.71	14.80
Mg (mg kg^{-1})	163.07	114.16	212.16	223.79
Fe (mg kg^{-1})	6.06	3.26	10.71	9.01
Mn (mg kg^{-1})	7.60	4.08	7.90	3.98
Zn (mg kg^{-1})	1.92	2.22	1.79	2.17
Cu (mg kg^{-1})	0.44	0.33	0.25	0.14
Ni (mg kg^{-1})	0.25	0.12	0.21	0.12
B (mg kg^{-1})	0.07	0.03	0.06	0.04

Table S4 Best-fitted GAM models including each set of environmental explanatory variables (geoclimatic factors, edaphic properties and soil fertility) and their combinations as predictors of *Tuber melanosporum* mycelium biomass. Separate models were fitted considering the entire dataset, and the separate datasets of plantations and forests. The final models were selected by FSSgam package. Based on the deviances explained (DE), the relative contribution of each model scenario was quantified as indicated in the sixth column. AIC = Akaike Information Criterion.

Response variable	Explanatory variables (Abbreviation)	All samples		Plantations		Forests		Relative contribution quantification	All samples	Plantations	Forests
		DE %	AIC	DE %	AIC	DE %	AIC		Relative DE %		
T. melanosporum soil mycelium	Geoclimatic (G)	41.1	1667.2	68.5	788.7	43.6	843.3	$DE_{GEF} - DE_{EF}$	-0.4	-0.6	-6.2
	Edaphic (E)	44.0	1658.9	70.1	781.9	53.7	840.1	$DE_{GEF} - DE_{GF}$	1.2	-2.4	1.3
	Fertility (F)	44.2	1659.9	68.1	781.9	57.4	834.5	$DE_{GEF} - DE_{GE}$	1.5	-2.9	2.3
	Geoclimatic_Edaphic (GE)	43.1	1658.5	70.5	781.7	47.0	839.3	$DE_{EF} + DE_{GF} - DE_{GEF} - DE_{F}$	-0.5	2.4	-3.2
	Geoclimatic_Fertility (GF)	43.3	1649.5	70.0	779.7	48.0	836.4	$DE_{EF} + DE_{GE} - DE_{GEF} - DE_{E}$	-0.5	0.9	-0.5
	Edaphic_Fertility (EF)	45.0	1653.6	68.1	781.9	55.5	835.4	$DE_{GF} + DE_{GE} - DE_{GEF} - DE_{G}$	0.7	4.4	2.1
	Geoclimatic_Edaphic_Fertility (GEF)	44.5	1647.2	67.5	780.5	49.3	834.3	$DE_{GEF} + DE_{G} + DE_{E} + DE_{F} - DE_{GE} - DE_{GF} - DE_{EF}$	42.6	65.7	53.4

Chapter 2

Tuber melanosporum Vittad. abundance and specific soil parameters predict soil enzymatic activity in wild and managed truffle producing systems

Submitted to:
Applied Soil Ecology

Vasiliki Barou, Mario Zabal-Aguirre, Javier Parladé, Ana Rincón



Abstract

Soil microorganisms are able to produce extracellular enzymes and are crucial for ecosystem processes like decomposition and nutrient cycling. The black truffle (*Tuber melanosporum* Vittad.) is a highly valued ectomycorrhizal edible fungus. It exerts a strong allelopathic effect, creating a burnt area around the host tree that impacts soil biotic and abiotic properties, and likely affects soil functioning as well. This study investigated the influence of black truffle abundance on soil functions, at different seasons and truffle-producing systems. A regional field design was set up in black truffle productive plantations and forests across the natural distribution area of this fungus in Spain. Physico-chemical soil properties, potential soil enzymatic activities related with carbon, phosphorus and nitrogen cycling, and soil fungal richness and abundance were determined. Black truffle-producing forests generally exhibited lower enzymatic activity compared to plantations, except for chitinase. Besides greater soil enzymatic activity —mainly related with rapid carbon and nitrogen turnover— was observed in late spring than autumn, independently of the type of truffle producing system. Our findings revealed a significant negative impact of black truffle abundance on soil enzymatic activities, and particularly on those involved in carbon and nitrogen mobilisation. Besides the strong local site effect, other biotic and abiotic factors differently impacted soil functioning in truffle forests (Mg) and plantations (Ascomycetes richness, $CaCO_3$, Na). These results offer insights into the ecology and functionality of host-truffle-soil interactions and provide valuable information for optimising management practices in black truffle plantations.

Keywords: soil enzymatic activity; carbon cycling; nutrient cycling; soil fungi; *Tuber melanosporum*; black truffle producing systems

Highlights

- Black truffle abundance has a negative effect on soil nitrogen mobilisation
- Soil enzymatic activity is lower in truffle-producing forests than plantations
- In black truffle systems, soil enzymatic activity is higher in spring than autumn
- **Soil** properties differently impact soil function in truffle forests and plantations

1. Introduction

Soil microorganisms are main drivers of soil organic matter (OM) formation, by their ability to release extracellular enzymes that gradually decompose the plant litter, root exudates, and microbial necromass (Baldrian, 2014; Maillard et al., 2021). Through this important ecosystem process, the macromolecules of litter are transformed into soluble compounds that supply microorganisms and plants with energy and nutrients, promoting, in this way, their growth (Chapin et al., 2011). As the rate of litter breakdown regulates the amount and timing of carbon and nutrients stored in soil and released to the atmosphere, decomposition plays a crucial role in the global carbon and nutrient cycling (Bonanomi et al., 2023). Soil enzymatic activity is linked to the rate of litter breakdown, and is a good indicator of soil health because it is closely related to soil fertility and quality (Adetunji et al., 2017; Hassan et al., 2022). Assessing soil enzymatic activity is thus considered to provide important information for the biological assessment of soil function (Alrumman et al., 2015).

The decomposition rate regulating factors are both biotic and abiotic, and mainly related with the litter quality, the environmental conditions and the microbial community involved in the process (Allison, 2006; Berg & McClaugherty, 2020). In fact, soil enzymatic activity is affected by soil microorganisms, mostly by their taxonomic composition and biomass (Chapin et al., 2011), pointing to the microbial carbon and nutrient demand as important drivers of soil OM decomposition (Sinsabaugh et al., 2008). Soil microbial communities are strongly affected by the land use (Thomson et al., 2015; Creamer et al., 2016; Xu et al., 2023; Devi et al., 2024) that also affects important soil processes e.g., carbon and nutrient cycling (Kidinda et al., 2023). The land use of an ecosystem determines the amount and type of litter produced and its susceptibility to decomposition (Chapin et al., 2011; Peña-Peña & Irmler, 2022; Zhang et al., 2023) and thus, the organic matter input to the soil. For example, the litter of perennial trees in a plantation is likely to decompose rapidly, while the litter of mixed deciduous tree species in wild forests is accumulated and decomposed slowly (Fanin et al., 2016). Litter quantity and quality significantly influences soil nutrient cycling, with its recalcitrance depending on the production of polyphenols by specific tree species (Hättenschwiler & Vitousek, 2000). In addition, the response of soil enzymes to plant and microbial litter inputs is highly controlled by edaphic and climatic factors (Ai et al., 2023). In fact, seasonality is another important factor influencing soil enzymatic activity, which is usually enhanced during the plant's growing season, from fall to early spring, and decreases in summer (Buée et al., 2005; Baldrian et al., 2013; Kivlin & Treseder, 2014). This indirect effect is explained by the increased soil moisture and substrate availability along with the growing season that stimulate microbial activity (Parker & Schimel, 2011). Seasonal effects may be particularly relevant in warm and water-limited Mediterranean ecosystems, which are highly

vulnerable to climate change (Scarascia-Mugnozza et al., 2000; Vogel et al., 2021). The complexity of all the above-mentioned ecological interactions evidence that soil processes can be highly dependent on the specific biotic and abiotic ecosystem characteristics. Analysing the intricate relationships between soil enzymatic activity and its key influencing factors is thus crucial for gaining a deeper understanding of soil carbon and nutrient cycling, and, therefore, ecosystem functioning.

In this study, we focused on Mediterranean ecosystems that produce black truffle under different land use conditions (i.e., forests and agro-forestry systems), where the dominant fungal species is *Tuber melanosporum* Vittad. Comparing soil processes in natural and managed systems may provide valuable information on the ecology of host-truffle-soil relationships. The dominance of black truffle in these systems has been often related with reduced plant and microbial diversity, and it becomes apparent in the areas around truffle-colonised trees with scarce vegetation, the so-called "brûlés" (Streiblová et al., 2012; Mello et al., 2015; Schneider-Maunoury et al., 2018). *Tuber melanosporum* mycelium biomass has been positively correlated with the expansion of the brûlé area (Queralt et al., 2017), indicating a potential influence of its abundance on the above- and belowground organisms. On the sight of the dominant effect of *T. melanosporum* observed in the brûlés, it is worth wondering whether these biotic interactions can affect the soil functioning through the enzymatic activity regulation. The studies of black truffle genome have revealed that this fungus exhibits several genes coding for cell wall degrading enzymes (Martin et al., 2010; Balestrini et al., 2012). In addition, despite the reduced biotic diversity within the truffle brûlé, a strong metabolic activity has been reported (Zampieri et al., 2016). Recent studies on *Tuber aestivum* Vittad. orchards have evidenced the relationship between soil enzymatic activity and the fungal presence and truffle production (Innangi et al., 2020; Bragato et al., 2021). However, to the best of our knowledge, the effect of *T. melanosporum* on soil functioning (i.e., enzymatic activity) has not been studied before, and this was the primary objective of this work.

We first hypothesised that the soil functioning was influenced by *T. melanosporum*, whose abundance would explain the activity of enzymes related with carbon (C), phosphorus (P) and/or nitrogen (N) cycling in soil (hypothesis 1). We also anticipated higher enzymatic activity related to C and N mobilisation in truffle-productive forests compared to plantations, given the higher soil organic matter content (Barou et al., 2024) and the greater conservative N use reported in forests (Averill et al., 2019) (hypothesis 2). The relationships of other physical-chemical soil parameters with the enzymatic activity inside the brûlé were also investigated. Active plant's growth together with edaphic-climatic conditions are key drivers of soil microbial activity (Francioli et al., 2018; Xu et al., 2021; McBride et al., 2023), thus we also expected an enhanced soil enzymatic activity related with

the mobilisation of key nutrients for plants and microorganisms such as N and P in spring compared with autumn (hypothesis 3).

To test these hypotheses, we analysed a set of soil enzymatic activities (i.e., as a proxy of soil functioning) within the black truffle brûlés, in two different truffle producing ecosystems, i.e., plantations and forests, and at two seasons, i.e., autumn and late spring. This comparative analysis will provide valuable clues on black truffle ecology, which can be useful to design management practices while enhancing soil ecosystem services in truffle producing plantations.

2. Materials and Methods

2.1. Study area and sampling

We selected 18 sites located along the natural distribution area of the black truffle in Northeastern Spain, including two types of truffle production: a) planted black truffle orchards (17 sites) and b) wild black truffle woodlands (10 sites) (**Fig. 1**, see Barou et al., 2024 for more details). All plantations are truffle producing oak monocultures over 10 years old, where standard agriculture management is currently conducted (i.e., soil tillage, irrigation, pruning). In forest sites, *Quercus ilex* L. dominated the uppermost canopy layer while the understory had species such as *Quercus faginea* Lam., *Juniperus* spp., *Cistus albidus* L., *Crataegus monogyna* Jacq., *Rosmarinus officinalis* L. and *Thymus* spp. The study areas show continental to Mediterranean climate with seasonal hydroclimatic variations, and the mean altitude of the sites is 936 ± 251 (SD) m (a.s.l.). The mean annual temperature for the period 2010-2018 is 12.3 ± 1.3 °C and the mean annual precipitation is 546 ± 128 mm (Fick & Hijmans, 2017).

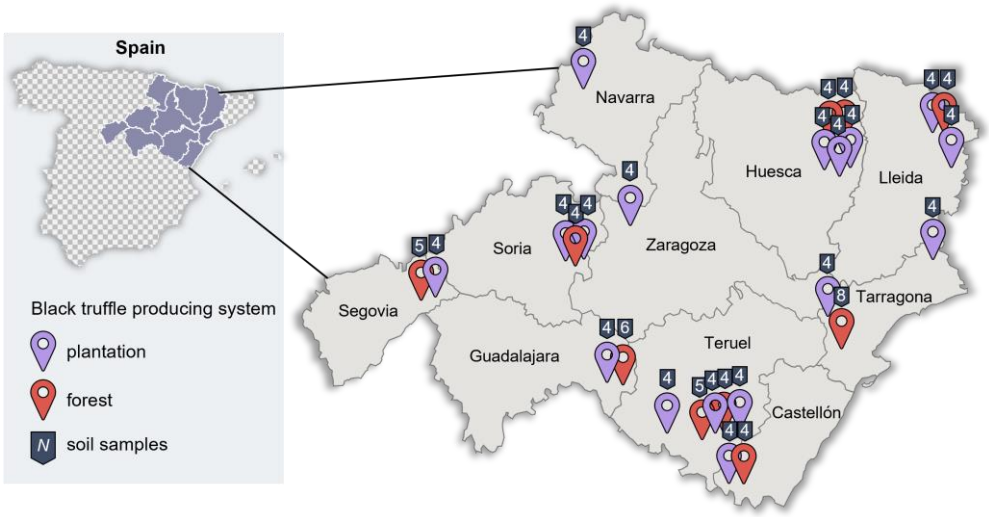


Figure 1 Location of black truffle producing plantations (purple) and forests (red) across the natural distribution area of this fungus in Spain, where soil samples were collected. The numbers represent holm oak trees sampled in each site. Map created with flourish.studio (https://flourish.studio) and adapted by the authors.

In each site, we selected at least four productive *Q. ilex* trees for sampling soil, to account for the plot heterogeneity, and we sampled all sites in autumn 2019 and spring 2020. A total of 116 holm oaks were sampled at both seasons (68 trees in plantations and 48 in forests), with 232 soil samples processed in total. All trees had been previously verified to be truffle-productive, by confirmation of the plantation owners and the forest truffle hunters.

For soil sampling, after removing the litter, we used a metallic probe to obtain soil cylinders of 4 cm diameter and 20 cm deep. A total of four samples per tree were collected from the four orientations at the middle point between the tree trunk and the limit of the brûlé, when this was visible, or the projection of the tree crown when it was not. The four samples from each tree were pooled into a unique soil sample per tree and stored in plastic bags at 4 °C until processing.

2.2. Physical-chemical soil properties and enzymatic tests

The soil samples were homogenised by hand and prepared for analysis. Soil stoniness, soil texture (% sand, clay and silt), gravimetric soil moisture (GSM), pH, electrical conductivity (EC), organic matter (OM), calcium carbonate ($CaCO_3$), active carbonate, extractable phosphorus (P), total N, extractable potassium (K), magnesium (Mg), calcium (Ca) and sodium (Na), iron (Fe), zinc (Zn), manganese (Mn), boron (B) and copper (Cu) were measured as described in Barou et al. (2024). Soil parameters were determined for the sampled soils at both seasons, except for texture variables and $CaCO_3$ that were only measured for the samples of autumn 2019.

Soil functioning was evaluated by measuring potential activities of eight hydrolytic and oxidative exoenzymes secreted by microorganisms: β-glucosidase (EC 3.2.1.21; ExPASY database, https://enzyme.expasy.org) and β-cellobiohydrolase (EC 3.2.1.91) that degrade cellulose, β-xylosidase (EC 3.2.1.37) and β-glucuronidase (EC 3.2.1.31) that degrade hemicellulose, alkaline phosphatase (EC 3.1.3.1) involved in the mobilisation of phosphorus, chitinase (EC 3.2.1.14) which hydrolyses chitin, leucine aminopeptidase (3.4.11.1) related to the mobilisation of nitrogen from peptide substrates and laccase (1.10.3.2) activity, which is involved in lignin degradation. To measure the potential soil enzymatic activity, we followed the methodology of Courty et al. (2016) and Pérez-Izquierdo et al. (2017). Briefly, 1 g of soil was incubated overnight (100 rpm, at 25 ºC) in the buffer (Tris-maleate 40 mM, pH 8 for leucine; Tris-acetate 10 mM, pH 11 for alkaline phosphatase; and Tris-acetate 10 mM, pH 4.5 for the rest of enzymes). Based on fluorogenic substrate release, the enzymatic activity for all enzymes except laccase was determined by using methylcoumarin (AMC) for leucine aminopeptidase and methylumbelliferone (MU) for the rest of enzymes. The laccase was determined by a photometric assay based on the ABTS substrate (2,2'-Azino-bis (3-ethylbenzo-thiazolin-6-sulfonic acid)). To measure enzymatic activities, a Victor X3 multimode plate reader (PerkinElmer Inc., Massachusetts,

USA) was used, at wavelengths of excitation/emission of 355/460 nm for fluorogenic assays and 415 nm for laccase activity. Results were expressed at pmol x mg soil^{-1} x min^{-1} x OM^{-1}.

2.3. Molecular and bioinformatics analyses

For each sample, we carried out DNA extraction from 0.25 g sieved dry soil using DNeasy Power Soil Pro HTP 96 kit (Qiagen, Germany). To estimate the abundance of *T. melanosporum* and companion fungi, metabarcoding analysis was performed targeting the fungal ITSI-5.8S region of rDNA with the primers ITS1F/ITS2 (Gardes & Bruns, 1993). Blank samples were produced at DNA extraction and PCR steps to control for possible contaminants. PCR amplicons were sequenced using Illumina MiSeq, at the IBLN-CSIC sequencing service (Granada, Spain). Due to sequencing failure in one sample, the final dataset consisted of 231 samples.

To test if the number of sequences of *T. melanosporum* was a good proxy of its abundance, black truffle myceliar biomass (µg of mycelium g^{-1} of soil) was measured by a real-time Taqman® PCR, as previously described (Parladé et al., 2007, 2013). Fit abundance estimates of *T. melanosporum* obtained by the two methods i.e., NGS-sequence and qPCR-mycelial biomass, Spearman's rank correlation (*Rho* = 0.84, $p < 0.001$) and polynomial regression ($R^2 = 0.84$, $p < 0.001$), showed a significant and quite precise relationship of the log-transformed measures (**Fig. S1**).

The demultiplexed NGS-sequence set (15,580,283 reads) was processed, and clustered into operational taxonomic units (OTUs) using the DADA2 bioinformatics pipeline (Callahan et al., 2016; R Core Team, 2022). Primers were removed with the software cutadapt v.3.4 (Martin, 2011), and after filtering and trimming, de-replication, denoising, merging and chimera identification steps, a matrix with amplicon sequence variants (ASV) and their frequency per sample (i.e., proxy of abundance) was obtained. Fungal taxonomic categories were assigned to ASVs using the RDP Naive Bayesian Classifier algorithm (Wang et al., 2007) implemented in DADA2 with a bootstrap threshold of 80 % using the UNITE v8.2 database (Abarenkov et al., 2020)**,** and clustered at 97% similarity into OTUs using VSEARCH v.2.17 (Rognes et al., 2016). OTUs were further curated by applying the LULU algorithm (Frøslev et al., 2017). A final matrix of 8,654 fungal OTUs and 7,151,228 reads was obtained, after removing OTUs with less than two reads. The database was normalised by dividing the number of sequences per OTU in a sample by the total number of sequences of that sample.

2.4. Statistical analyses

In order to test whether *T. melanosporum* abundance, the type of truffle-producing system (hereafter “type”), and the season of sampling (hereafter “season”) explained soil functioning, linear

mixed models (LMM) were fitted for each enzymatic activity with these fixed factors, with the site as random factor, by using the *lmer* function of "lme4" R package (Bates et al., 2015). Soil enzymatic activities were log- or square root-transformed and the distribution of residuals was examined to test normality with the Shapiro test ($p > 0.05$). The marginal and conditional R-squared was computed to calculate the proportion of variance explained by fixed factors and by both fixed and random factors, respectively using the *r.squaredGLMM* of "MuMIn" R package (Bartoń, 2015). In addition, pairwise comparisons of the levels of the categorical factors "type of producing system" (forest, plantation) and "season" (spring, autumn) were performed using *lsmeans* function of "lsmeans" package (Lenth, 2016). To visualise the marginal effects of *T. melanosporum* on enzymatic activities the *ggpredict* function of "ggeffects" R package (Lüdecke, 2018) was used. The abundance of *T. melanosporum* was also tested for significant differences among the levels of type and season, applying LMM and setting the site as random factor. Interactions between type and season were initially included in all models but they were not significant and were thus excluded from the final models.

To analyse the influence of soil and fungal parameters on soil carbon and nutrient cycling inside the brûlé in plantations and forests, we performed redundancy analyses (RDA) with the enzymatic activities as dependent variables and a range of explanatory variables, i.e., season and site of sampling, *T. melanosporum* abundance, richness and abundance of the rest of fungi (also including separately Ascomycetes and Basidiomycetes) and soil abiotic parameters (29 variables in total, see the list in **Table S1**). RDAs were separately performed for plantation and forest datasets. Previous Spearman correlation analyses with soil and fungal variables were done to check their possible relationships. To find significant explanatory variables for the potential soil enzymatic activity, a forward model selection was carried out with *ordiR2step* function of "vegan" R package (Oksanen et al., 2022). The collinearity between the selected explanatory variables of the resulting RDA model was checked through variance inflation factors (VIFs) with *vif.cca* function. Any variables with VIF > 10 were excluded from the models (Kim, 2019). The variance explained by each variable was obtained from permutation-based ANOVA with *anova.cca* function. The ordination was performed using the *rda* function of "vegan" R package with the Euclidean distance matrix of enzymatic activities and the visualisation of the first two axes of RDA with *ggord* function of "ggpubr" R package (Kassambara, 2022).

All analyses were performed with the R software 2022 v.4.2.1 (R Core Team, 2022).

3. Results

On average, the abundance of *T. melanosporum* was significantly higher in plantations than in forests ($F = 45.82$, $p < 0.001$), as well as in autumn than in spring ($F = 22.12$, $p < 0.001$) (**Fig. S2a**). Across all treatments, *T. melanosporum* was the dominant fungal taxon, representing on average 12 - 22 % of total reads, while the next four most abundant taxa represented on average 0.1 - 8.3 % of total fungal abundance (**Fig. S2b**). In fact, *T. melanosporum* abundance negatively correlated with that of the rest of fungi, in wild and managed truffle-producing systems, and also with overall fungal richness in plantations (**Fig. S3**). In both systems, black truffle abundance was strongly and positively correlated with Ascomycetes abundance, and strongly and negatively correlated with Basidiomycetes abundance (and also richness in forests) (**Fig. S3**).

3.1. Effect of T. melanosporum *abundance, the type of producing system and the season on potential soil enzymatic activities inside the brûlé*

As expected, *T. melanosporum* abundance did have a significant effect on the potential soil enzymatic activity, particularly of enzymes related with the C-cycle such as β-xylosidase (marginal) and β-glucuronidase, as well as with chitinase and leucine, involved in N cycling (**Fig. 2**; **Table S2**). In all cases, the relationship between *T. melanosporum* abundance and these enzymatic activities was negative (**Fig. 2**).

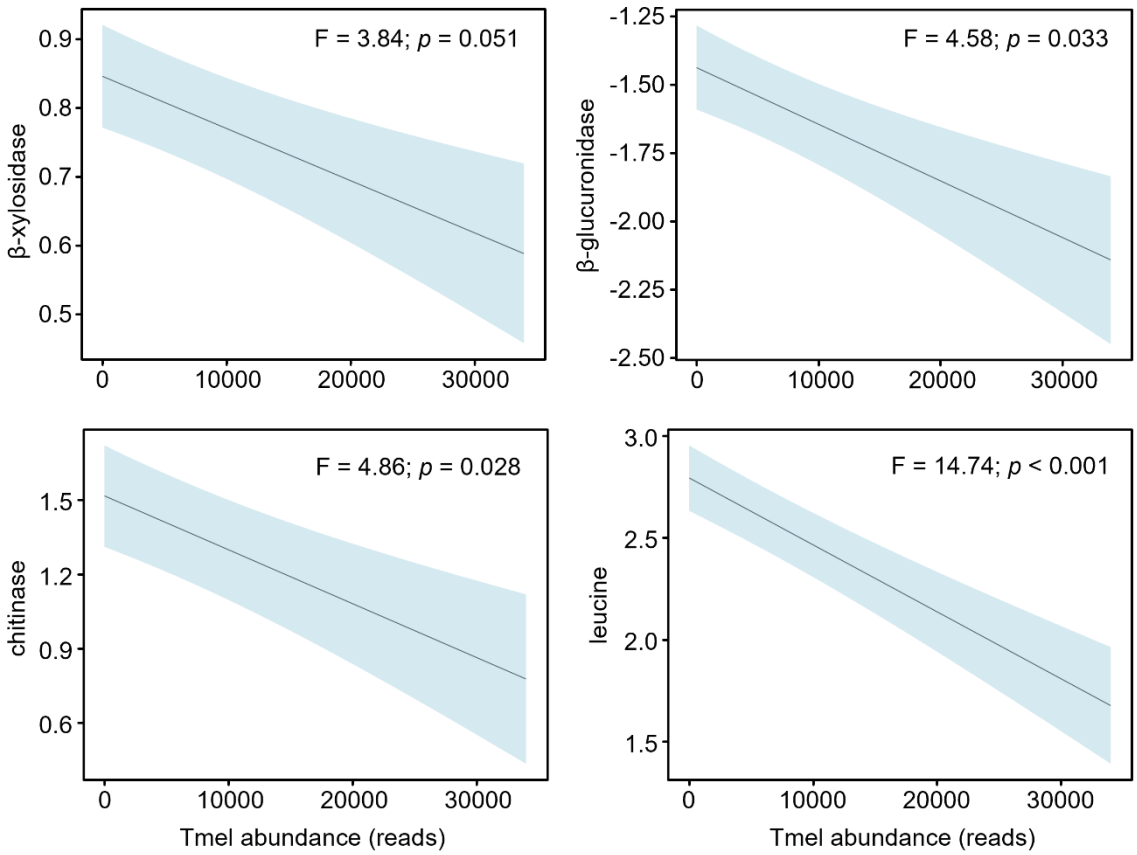


Figure 2 Relations (mean predicted values) between *Tuber melanosporum* (Tmel) abundance and the potential soil enzymatic activities. The relationships were estimated by linear mixed models (see **Table S2**), with the site as random factor. Tmel measured as sequencing reads; enzymatic activities were log- or sqrt- transformed and measured as pmol x mg soil^{-1} x min^{-1} x soil organic matter (OM)$^{-1}$ The blue area in all graphics represents the 95 % confidence intervals (*N* = 231).

The alkaline phosphatase activity, involved in phosphorous mobilisation, was significantly higher in plantations than forests, as well as the laccase activity involved in recalcitrant C degradation (**Fig. 3; Table S2**). However, the opposite occurred with the chitinase activity that was higher in forests than plantations (**Fig. 3**; **Table S2**). On average, the activity of β-glucosidase, β-xylosidase (C-related) and chitinase (N-cycling related) was significantly higher in spring than autumn (**Fig. 3**), independently of the type of truffle producing system.

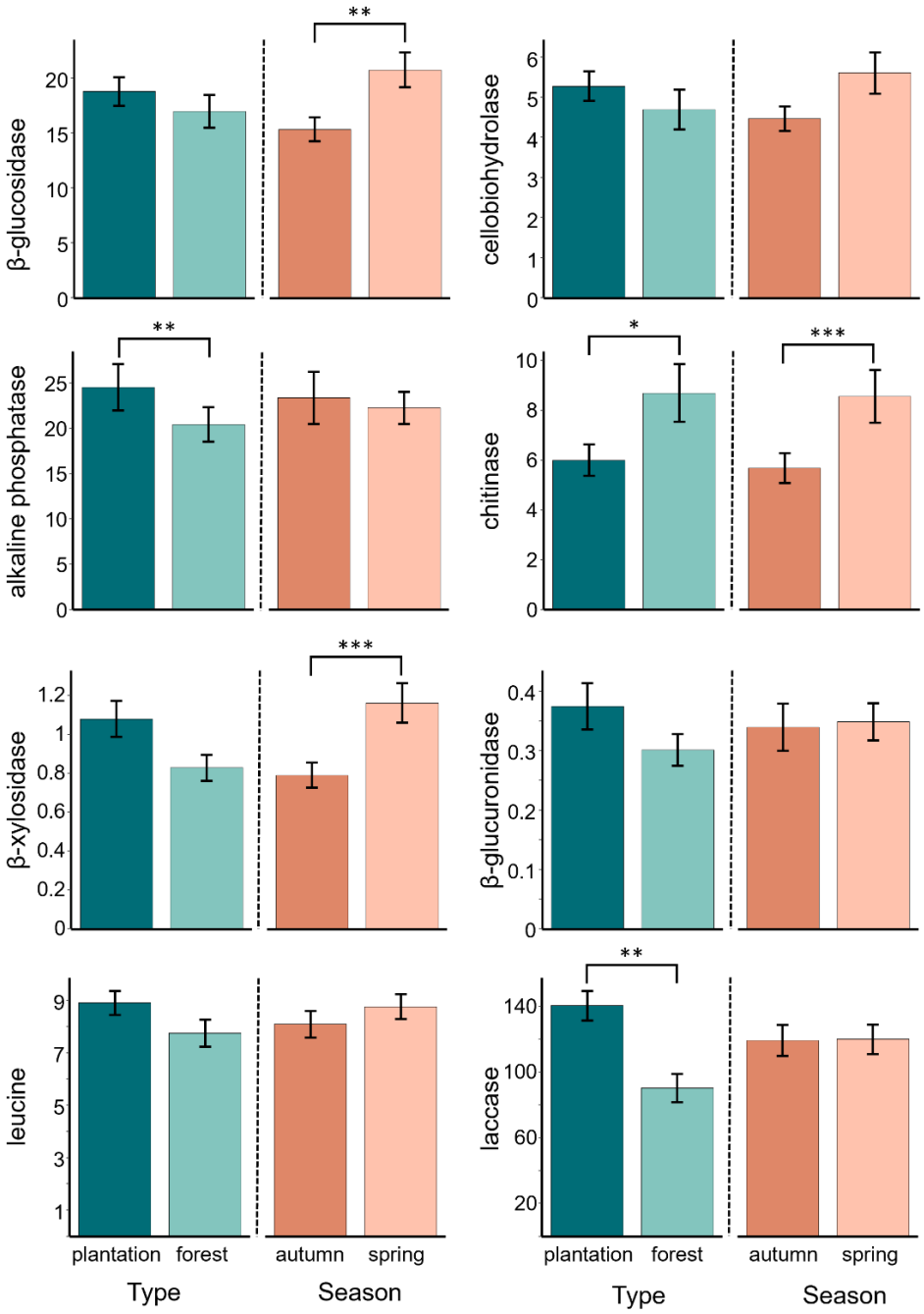


Figure 3 Potential soil enzymatic activities (mean ± SE) across the different types of truffle producing systems and seasons of sampling. Linear mixed models (LMMs) were fitted with the type (plantations *N* = 135 and forests *N* = 96), season (autumn *N* = 116 and spring *N* = 115) and *Tuber melanosporum* abundance (reads) as fixed factors, and the site as random factor (see **Table S2**). Significant differences among the treatments of each factor "type" or "season" are indicated by asterisks: *p<0.05, ** p<0.01; ***p<0.001. Enzymatic activities were measured as pmol x mg soil^{-1} x min^{-1} x soil organic matter (OM)$^{-1}$.

3.2. Main drivers of carbon and nutrient cycling inside the brûlé in forests and plantations

The RDA and forward model selection analyses showed that the abiotic and biotic predictors studied explained approximately 45 % and 41 % of the variance of potential soil enzymatic activities inside the black truffle brûlé in plantations and forests, respectively (**Table 1**). The first two axes (RDA1 and RDA2, out of a total of 8 axes) of each RDA represented 74 % and 77 % of the previous variance for plantation and forest datasets, respectively, thus covering well the information of the RDA models (**Fig. 4**). In both truffle-producing systems, the site of sampling accounted for the highest part of the explained variance of soil enzymatic activity (**Table 1**), and the abundance of *T. melanosporum* was selected as a significant predictor in both RDA models, confirming our previous results (see **Fig. 2**). Regarding other soil abiotic and biotic parameters, the $CaCO_3$ and Na content together with Ascomycetes richness were significant predictors of soil enzymatic activity inside the truffle brûlés of plantations, while Mg content together with the season, did significantly explain soil-brûlé potential enzymatic activity in forests (**Table 1**; **Fig. 4**). The B content, although chosen by the RDA forward selection in plantations, was not a significant explanatory variable of soil enzymatic activity variation (**Table 1**); Mg content, also chosen by forward selection in plantations, was discarded from the final model due to high collinearity (VIF > 10).

Table 1 Variance explained (%) by the predictors of soil enzymatic activities in truffle producing plantations (*N* = 135) and forests (*N* = 96), analysed by redundancy analyses (RDA). Predictors were chosen though the forward model selection in RDA among 29 potential explanatory variables including season and site factors (see **Table S1**; **Fig. S3**). ASCO = Ascomycetes; Tmel = *Tuber melanosporum*.

Plantations					**Forests**				
Predictor	Df	F	*p*-value	Variance (%)	Predictor	Df	F	*p*-value	Variance (%)
site	16	4.9	**0.001**	37.82	site	9	4.934	**0.001**	31.69
Tmel abundance	1	3.635	**0.008**	1.75	Tmel abundance	1	5.901	**0.001**	4.21
ASCO. richness	1	3.328	**0.017**	1.61	season	1	2.756	**0.024**	1.97
$CaCO_3$	1	3.37	**0.017**	1.63	Mg	1	4.051	**0.009**	2.89
Na	1	3.311	**0.028**	1.6					
B	1	2.229	0.063	1.08					
Sum				45.48	Sum				40.76
Residual	116			54.52	Residual	83			59.24

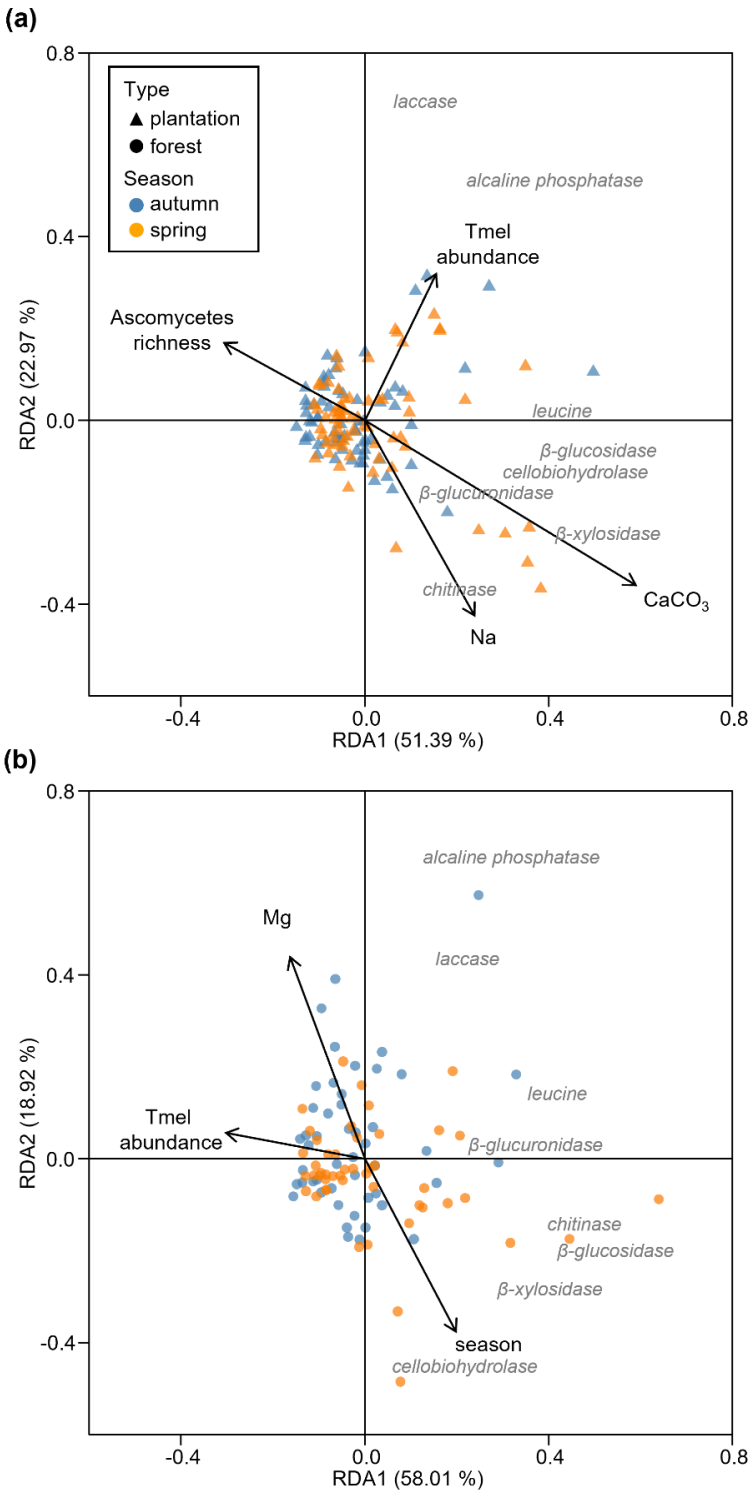


Figure 4 Significant predictors of potential soil enzymatic activities (grey) in black truffle brûlés of (a) plantations and (b) forests. Data were analysed by redundancy analysis (RDA) and forward step selection using the respective enzymatic activity matrix and a total of 29 abiotic and biotic explanatory variables including season and site factors (see **Table S1**; **Fig. S3**). The axes percentages are relative to the total amount of variance explained by the model (45,48% in plantations and 40,76% in forests) (**Table 1**). The length and direction of vectors indicate the strength of their relation with the ordination axes. Tmel = *Tuber melanosporum*.

4. Discussion

Regarding the *T. melanosporum* quantification methods (both targeting the ITS1 rDNA region), although the primer sets used in NGS and qPCR were different, the high correlation of the OTU-*T. melanosporum* and its absolute quantification though qPCR indicated that data from both methods are compatible for the quantification of this fungal species at the regional scale, as previously indicated in other local study (Garcia-Barreda et al., 2023).

4.1. Tuber melanosporum *abundance slows down soil enzymatic activities related to carbon and nitrogen mobilisation*

The abundance of *T. melanosporum* was significantly and negatively related with enzymes associated with N-cycling (i.e., chitinase and leucine), and C-cycling (i.e., β-xylosidase and glucuronidase), partially confirming our first hypothesis. This negative relationship may be linked to an indirect effect on soil microbial dynamics related with C and N mobilisation due to *T. melanosporum* pervasiveness. In *T. aestivum* plantations in Italy, the soil enzymatic activity was lower inside *versus* outside the brûlé, indicating the potential of the fungus in changing the chemistry and the microbial activity in the soil (Innangi et al., 2020). Although, in order to prove the black truffle effect in our study, a comparison between the areas where *T. melanosporum* dominates or not would be needed, previous studies have shown that the increase of *T. melanosporum* abundance was coupled with the decrease of abundance of soil fungi with saprotrophic and pathogenic life style, and/or other mycorrhizal fungi and bacteria (Domínguez Núñez et al., 2006; Mello et al., 2013; Bing et al., 2016; Mello & Zampieri, 2017). In addition, the significant effect of *T. melanosporum* abundance over all potential soil enzymatic activity, although explaining a moderate portion of the total variation (see RDA results, **Table 1**; **Fig.3**), was further shown when datasets of plantation and forests were separately analysed, indicating that this black truffle effect was consistent under different land-use regimes.

The significant effect of *T. melanosporum* abundance slowing down soil C and N cycling could be also related to the niche construction effect, recently proposed for this fungus (García-Montero et al., 2024). These authors suggested that the black truffle modifies the habitat for its own benefit, and generates a stressful environment (e.g., increasing carbonate weathering, exchangeable Ca^{2+} and active carbonate and, according to our study, also reducing C and N cycling) with detrimental consequences for the plant host nutrition. This truffle-altered soil environment would boost plant's C allocation to the roots, making more trophic sites available for truffle-ectomycorrhizae settlement.

Additional studies are needed to explore this theory further, and to validate the cascading effects of soil modifications mediated by black truffle on the host plant's physiological responses.

4.2. Land use and seasonal effects on potential soil enzymatic activities

Concerning the type of truffle production system, in contrast to what we assumed (hypothesis 2), most of the soil enzymatic activities were higher in plantations than in forests, although this difference was only significant for alkaline phosphatase and laccase. A possible explanation may be found in the threefold higher amount of available phosphorous (i.e., substrate) in plantations than in forests. Previous studies have confirmed the correlation of available P with alkaline phosphatase, and this enzyme is considered to play an important role in regulating the available soil P for microorganisms and plants (Margalef et al., 2017; Li et al., 2021). In addition, alkaline phosphatase activity may have been stimulated by irrigation applied in all the plantations, as suggested for greenhouse-raised crops (Wang et al., 2018). Lower P contents have been also observed in black truffle oak seedlings grown under water stress (Domínguez Núñez et al., 2009). In this context, precision irrigation techniques, as well as minimum mechanical soil disturbance, may result in faster nutrient recycling and soil quality improvement (Habig & Swanepoel, 2015; Zheng et al., 2024), which eventually may be beneficial for black truffle productivity in plantations. In addition, laccase activity was higher in plantations than forests, probably related with the different recalcitrance of organic matter (Theuerl & Buscot, 2010) and/or soil particle size distribution (Meng et al., 2024) in managed *vs* natural truffle-producing systems. Opposite to alkaline phosphatase and lacasse, chitinase activity was significantly higher in forests than in plantations. Chitinase activity in soil has been associated with OM content and N availability (Papa et al., 2014), as well as with microbial nutrient mobilisation from necromass (Frederiksen et al., 2013; Pérez-Pazos et al., 2024). Thus the higher OM content due to more diverse litter input (Zarafshar et al., 2020) may be at the origin explaining the differences in chitinase activity found in forests with respect to plantations. Decomposition could be slowed in forests soils compared with agroforestry ones by the differential representativeness of microbial guilds (likely dominated by saprotrophs in plantations), and the competition of mycorrhizal and saprotrophic fungi for nutrients (i.e., Gadgil effect, Fernandez & Kennedy, 2016; Choreño-Parra & Treseder, 2024). Further studies exploring decomposition rates, and the biotic and abiotic factors involved in truffle-producing ecosystems would be needed to better understand the functioning of soil in the brûlé.

According to our third hypothesis, a higher activity was observed in late spring than in autumn for β-glucosidase, chitinase (indicative of rapid C and N turnover, respectively) and β-xylosidase. In parallel, *T. melanosporum* abundance was lower in spring than autumn indicating the implication of

other biotic or abiotic actors in these season-related soil functional variations. In a *Q. ilex* forest in Southern Catalonia, Spain, β-glucosidase activity was also higher in spring than autumn (Sardans & Peñuelas, 2005), and the same pattern was observed for β-glucosidase and β-xylosidase in a forested stream in NE Spain (Mora-Gómez et al., 2016). By contrast, in Mediterranean pine-oak mixed forests, Prieto-Rubio et al. (2023) did not observe significant seasonal differences for a combination of enzymes related with C and N cycles. In our study, the optimal conditions of temperature and water availability during spring (Sardans & Peñuelas, 2005) coincide with the peak of vegetative growth and litter quantity of holm oak (Ogaya & Peñuelas, 2004), which probably resulted in a stimulated microbial growth (Franciolia et al., 2014) and subsequently increased enzymatic activity (Papa et al., 2014). Moreover, seasonality was a significant factor to explain the enzymatic activities altogether in RDA but only in forests, implying that it is a relevant factor to be further examined in unmanaged ecosystems, where no irrigation is applied.

4.3. Differential effects of biotic and abiotic soil properties on enzymatic activities in managed and natural truffle producing systems

Total Ascomycetes richness was found as a significant predictor of enzymatic activity but only in black truffle plantations, where this phylum was more diverse than in forests. In black truffle plantations in France, Mello et al. (2011) showed that, compared to outside the brûlé, the richness of Ascomycota was higher inside, representing the dominant phylum in the brûlé. Given that, it could be thought that the Ascomycota diversity may be somehow related with the higher abundance of *T. melanosporum* in plantations than in forests. Future studies focusing on the relationship between specific trophic guilds and soil enzymatic activity are needed (Eisenhauer et al., 2022) to unveil the complex interactions in black truffle brûlés.

In terms of abiotic soil properties, the contents of Na and $CaCO_3$ in soil were significant predictors of soil enzymatic activity, but only in plantations. Under Mediterranean conditions, Na has been associated positively with enzymatic activities in olive grove areas of Portugal (Reyes-Martín et al., 2023). A recent study in an intensive system with cereal crops has also showed that $CaCO_3$ affected significantly and, mainly in a positive way, soil phosphatase activities (Lin et al., 2023). In addition, $CaCO_3$ was considered as important driver of *T. melanosporum* mycelium (Barou et al., 2024), thus its relationship with enzymatic activities may be explained though the enhanced fungus abundance. According to the niche construction theory in *T. melanosporum* brûlés, in view of higher exchangeable Ca^{2+}, probably an ongoing modification of the total carbonate pool is taking place (García-Montero et al., 2024), which could thereby affect enzymatic activities, as we previously pointed out. Magnesium was also an important predictor of potential soil enzymatic activity (i.e., negative relationship).

Magnesium is well known for its role in chlorophyll synthesis and protein biosynthesis as a crucial co-factor for many enzymes (Senbayram et al., 2015). A short-term experiment in Mg-deficient soils showed that Mg fertilisation had a significant effect on soil enzymatic activities, acting as a potential regulator in nutrient cycling (Yang et al., 2021). A recent metagenomic analysis in saline-alkali soils in China suggested that that changes in Mg and also in Na contents in the soil could explain the microbial community variation, while Na additionally showed a significant effect on carbohydrate-active enzymes (CAZymes) encoding genes (Yin & Zhang, 2022).

Finally, it is important to note that, in both truffle-producing systems, local site conditions had a strong weight on soil enzymatic activity, and that almost half of the variance remained unexplained by the variables considered in our study, which indicates that other biotic (bacteria, soil fauna) and/or abiotic (soil structure, climatic conditions) actors are most likely implicated, as previously suggested (Eisenhauer et al., 2022; Perez-Lamarque et al., 2022). Further studies exploring the biotic and abiotic factors involved in soil processes such as carbon storage and nutrient mobilisation, as well as the direct and indirect relationships with truffle abundance and the host-tree performance are needed to better understand the ecology and functionality of truffle-producing ecosystems.

5. Conclusions

Our regional field study has revealed several key findings. First, the abundance of black truffle has a significant impact on soil enzymatic activity in both natural and managed production systems, alongside other specific biotic and abiotic soil factors unique to each system. On the other hand, the enzymatic activity in truffle producing forest soils is generally lower —specifically for alkaline phosphatase and laccase— compared to plantations, with the exception of chitinase that is involved in nitrogen mobilisation. Lastly, greater soil enzymatic activity related to rapid carbon and nitrogen turnover is observed in late spring compared to autumn, independently of the type of truffle producing system. Together all these results provide valuable information on the ecology and functioning of host-truffle-soil relationships and may have important implications to design optimal management practices (e.g., irrigation or fertilisation), while enhancing soil ecosystem services (i.e., nutrient cycling) in truffle producing plantations.

CRediT authorship contribution statement

Vasiliki Barou: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualisation, Writing – original draft, Writing – review and editing. **Mario Zabal-Aguirre**: Data curation, Methodology, Software, Validation, Writing – review and editing. **Javier Parladé**: Conceptualisation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing. **Ana Rincón**: Conceptualisation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – review and editing.

Declaration of Competing interest

The authors have no competing interests.

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Data availability

Data will be made available from the corresponding author upon reasonable request.

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7. Supplementary material

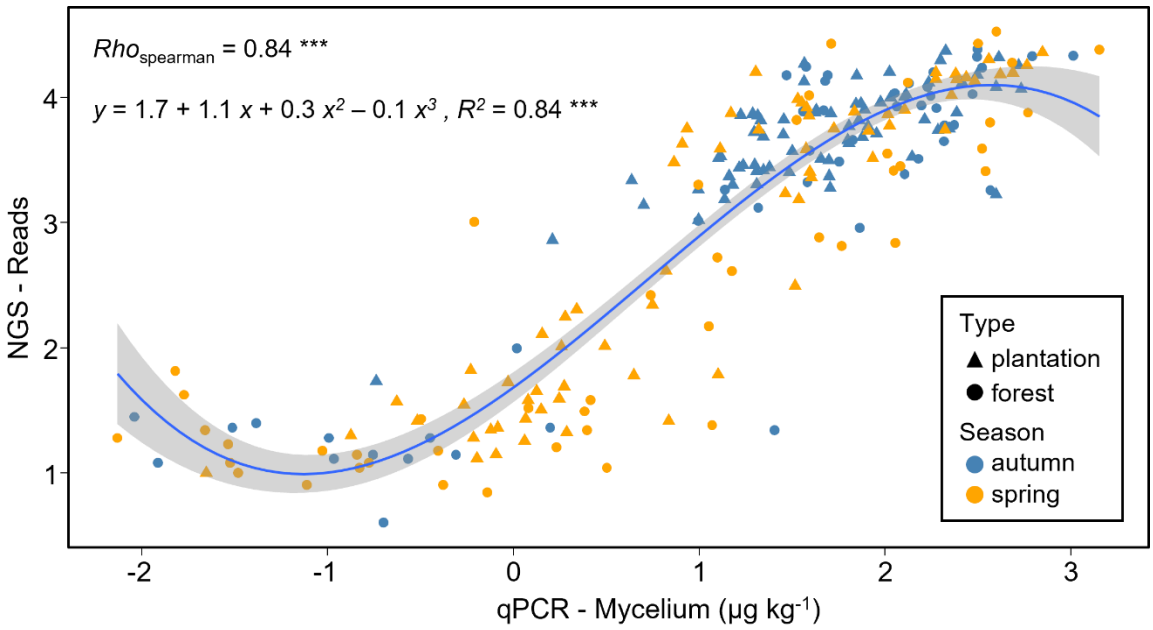


Figure S1 Fitting of *Tuber melanosporum* abundance estimates obtained by two quantification methods. Spearman correlation (Rho) and polynomial regression (R^2) were performed between the abundance (reads) of *T. melanosporum* (log-transformed), quantified though next-generation sequencing (NGS), and absolute quantity of *T. melanosporum* (log-transformed) soil mycelial biomass, quantified through RT-qPCR. *** p<0.001.

Table S1 Abiotic and biotic explanatory variables of potential soil enzymatic activity (dependent variable) used in redundancy analyses (RDA). Values are mean ± standard error (SE) from soil samples of truffle productive plantations and forests. GSM = gravimetric soil moisture, EC = electric conductivity, actCA = active carbonate, carb = $CaCO_3$, Tmel = *Tuber melanosporum*, ASCO = Ascomycetes, BASI = Basidiomycetes. *Categorical variable (season = autumn/spring, site = 18 sampled sites). Organic matter (OM) was the only variable not included as explanatory in RDAs because it was previously used to normalise soil enzymatic activity values.

Soil variable (units)	Plantations		Forests	
	Mean	SE	Mean	SE
pH	8.25	0.02	8.09	0.03
GSM (%)	11.99	0.3	11.27	0.57
EC (µs cm^{-1})	272.07	10.76	276.34	13.21
sand (%)	48.6	1.1	52.12	1.41
clay (%)	23.96	0.6	16.72	0.53
silt (%)	27.85	0.71	31.16	1.4
stone (%)	39.53	1.62	38.44	1.94
OM (%)	3.69	0.17	5.6	0.32
actCA (%)	6.57	0.36	4.94	0.33
carb (%)	28.48	1.59	39.2	2.59
N (%)	0.13	0.01	0.18	0.01
P (mg kg^{-1})	6.03	0.81	2.75	0.29
K (mg kg^{-1})	163.65	8.19	126.88	5.54
Ca (mg kg^{-1})	3297.1	78.94	3175.35	100.81
Na (mg kg^{-1})	14.99	1.73	9.71	1.51
Mg (mg kg^{-1})	151.41	9.37	212.16	22.84
Fe (mg kg^{-1})	5.99	0.25	10.71	0.92
Mn (mg kg^{-1})	8.22	0.35	7.9	0.41
Zn (mg kg^{-1})	1.93	0.19	1.79	0.22
Cu (mg kg^{-1})	0.46	0.04	0.25	0.01
B (mg kg^{-1})	0.07	0.00	0.06	0
Tmel abundance (reads)	5932	501.47	5613	807.52
Other fungi abundance (reads)	26078	655.13	23864	805.38
Other fungi richness (OTUs)	335	6.17	313	8.53
ASCO abundance (reads)	73	1	64	2.11
ASCO richness (OTUs)	213	3.92	200	5.28
BASI abundance (reads)	15	0.8	26	2.09
BASI richness (OTUs)	37	1.01	42	1.58
Season of sampling*	-	-	-	-
Site of sampling*	-	-	-	-

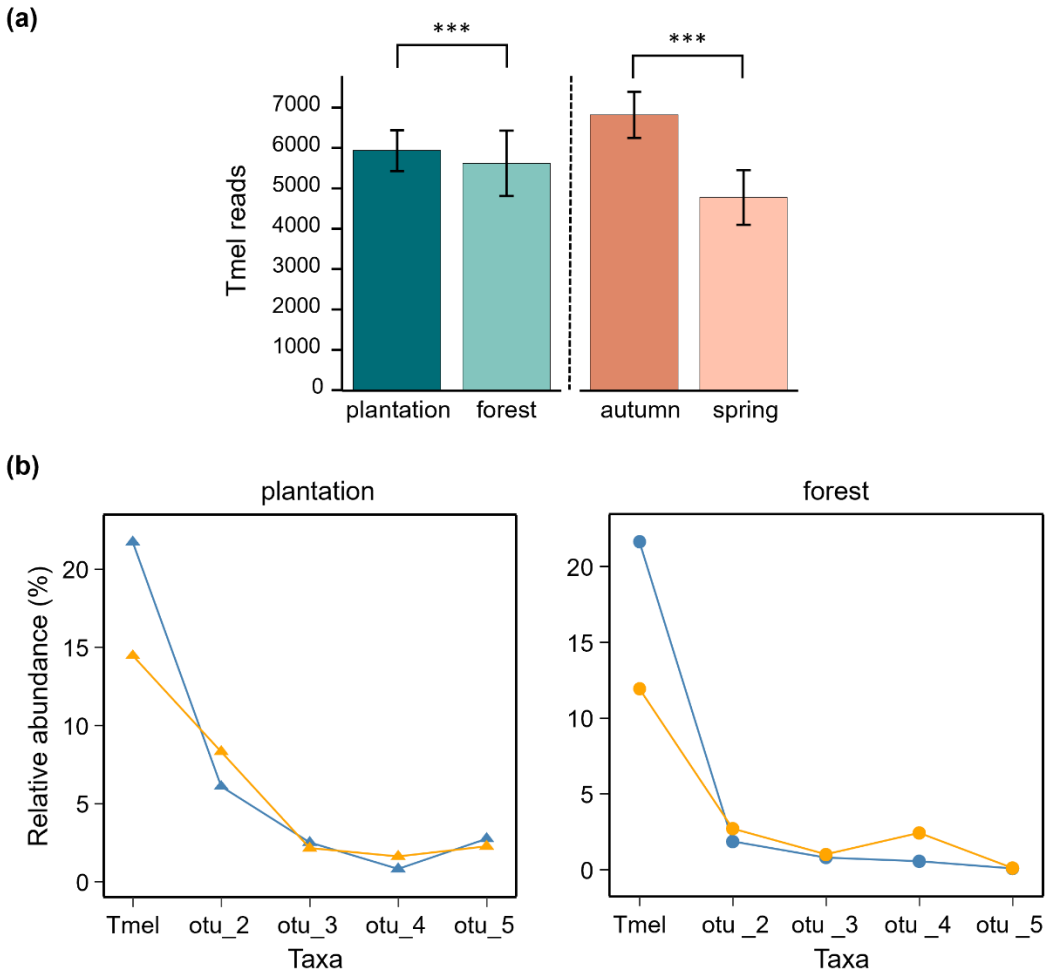


Figure S2 (a) Abundance of *Tuber melanosporum* (Tmel) by type of truffle production (plantations *N* = 135 and forests *N* = 96), and season of sampling (autumn *N* = 116 and spring *N* = 115). Tmel reads = *T. melanosporum* abundance estimated as sequencing reads. Bars represent mean ± standard error. Significant differences, depicted by asterisks (***p<0.001), among the levels of each factor were derived from linear mixed models fitted with *T. melanosporum* abundance (reads) as dependent variable, the type of truffle producing system and the season as fixed factors, and the site as random factor. (b) Mean relative abundance of the five most abundant fungal taxa across all treatments (blue = autumn, yellow = spring).

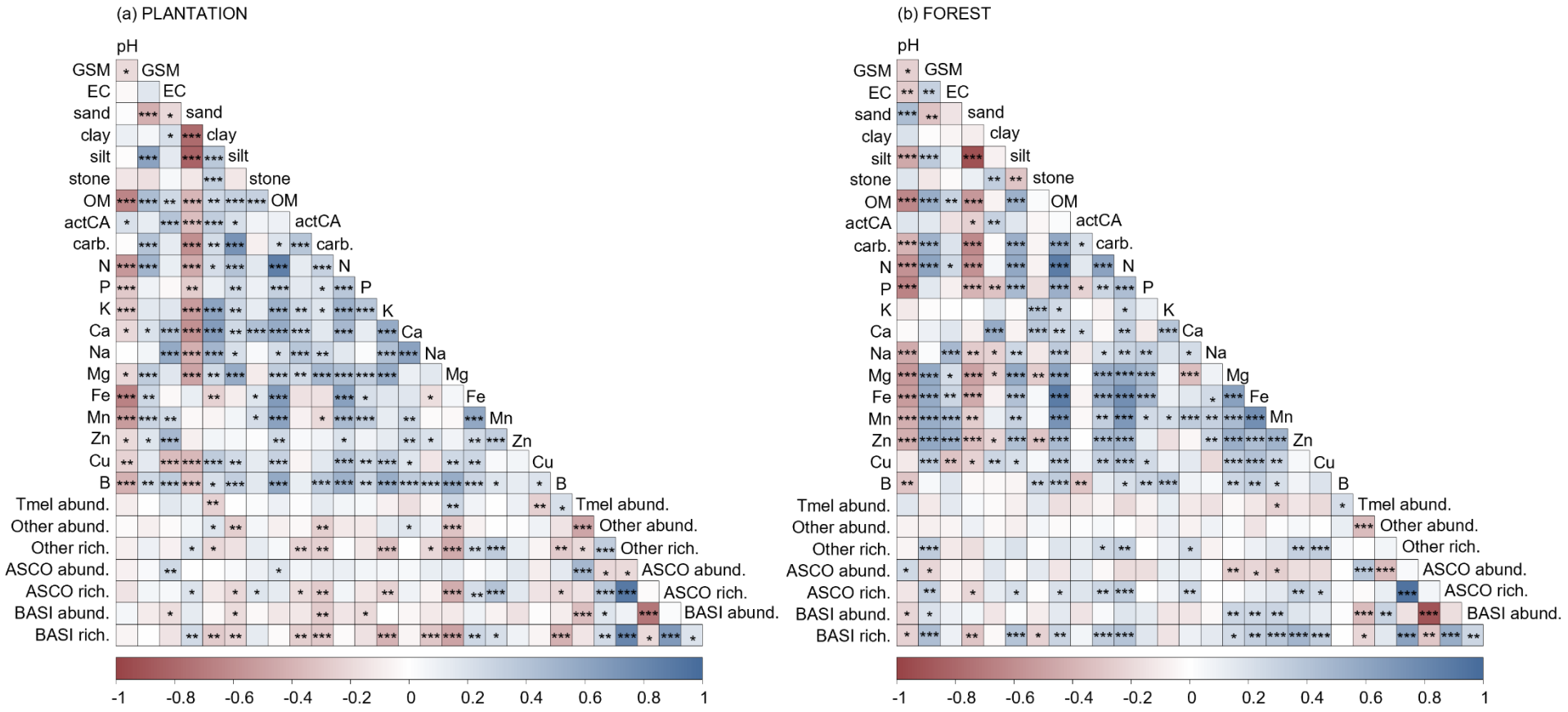


Figure S3 Spearman correlations among the abiotic and biotic explanatory variables of soils of black truffle brûlés of plantations (a) and forests (b), further used in the redundancy analyses (RDA) (**Fig. 4**; **Table S1**). GSM = gravimetric soil moisture, EC = electric conductivity, OM = organic matter, actCA = active carbonate, carb = $CaCO_3$, Tmel = *Tuber melanosporum,* Other = fungi other than *T. melanosporum*, ASCO = Ascomycetes, BASI = Basidiomycetes, abund. = abundance (reads), rich. = richness. Positive correlations are noted in blue and negative in red; significant correlations are noted by asterisks (*p<0.05, ** p<0.01; ***p<0.001).

Table S2 Linear mixed models fitted for potential soil enzymatic activities with the type of truffle producing system (type), the season and *Tuber melanosporum* abundance (Tmel reads) as fixed factors, and the site as random factor (N = 231). R^2m = marginal R squared; R^2m = conditional R squared. Enzymes were log- or square root-transformed to meet normality prior to models run. Significant effects ($p < 0.05$) of predictors are in bold.

Enzyme	Predictor	F-value	*p*-value	R^2m	R^2c
β-glucosidase	type	0.058	0.811	0.034	0.343
	season	9.431	**0.002**		
	Tmel	0.766	0.382		
cellobiohydrolase	type	0.604	0.438	0.013	0.21
	season	1.337	0.249		
	Tmel	0.939	0.333		
alkaline phosphatase	type	9.003	**0.003**	0.039	0.387
	season	0.432	0.512		
	Tmel	1.003	0.318		
chitinase	type	4.634	**0.032**	0.075	0.422
	season	12.694	**< 0.001**		
	Tmel	4.863	**0.028**		
β-xylosidase	type	1.096	0.296	0.058	0.352
	season	11.194	**0.001**		
	Tmel	3.84	**0.051**		
β-glucuronidase	type	0.315	0.575	0.027	0.153
	season	0.477	0.491		
	Tmel	4.58	**0.033**		
leucine	type	3.338	0.069	0.072	0.338
	season	0.087	0.769		
	Tmel	14.737	**< 0.001**		
laccase	type	7.521	**0.007**	0.03	0.364
	season	0.043	0.836		
	Tmel	0.162	0.688		

Chapter 3

Soil fungal community structure, network complexity and functional guilds in black truffle producing systems



1. Introduction

The colonisation and production success of black truffle (*Tuber melanosporum* Vittad.) has been extendedly studied in relation to the abiotic environment and its effects on the fungus´ growth (García-Montero et al., 2007; Garcia-Barreda et al., 2019; Barou et al., 2024). In the light of the niche construction theory, where black truffle is thought to have the ability to significantly modify the abiotic edaphic conditions (Bragato, 2014; García-Montero et al., 2024), soil biotic components could also be affected by black truffle in a shared niche. Under the same soil environment, in a process of dominating the space, the black truffle may show antagonistic interactions with the surrounding microbial communities. For instance, a competitive interaction has been found between *T. melanosporum* and other ectomycorrhizal fungi (ECM) for host plant and niche colonisation in brûlés, the areas around the hosts with scarce vegetation (Streiblová et al., 2012), as well as with other truffle species (Belfiori et al., 2012; De Miguel et al., 2014; Marjanović et al., 2020). Further potential competitive effects were also observed with arbuscular mycorrhizal fungi (AMF), molds, yeasts and plant pathogens, whose abundances have been negatively associated with that of *T. melanosporum* over time (Oliach et al., 2022).

Although the above-mentioned direct associations with other fungal guilds have provided some valuable insights regarding black truffle competitiveness, there is still a gap of knowledge on what is their role in the ecosystem function. For example, the competition between ECM and saprotrophic fungi in forest soils for water and nutrients has been considered to suppress decomposition rates, leading to greater carbon sequestration (Gadgil & Gadgil, 1971, 1975). However, this phenomenon is not consistent across the world, as it depends on several environmental and anthropogenic factors (Fernandez & Kennedy, 2016). On the other hand, ECM have been reported to interact with bacteria resulting in enhanced soil organic matter (OM) decomposition (Itoo & Reshi, 2013; Pérez-Izquierdo et al., 2019), and with AMF resulting in nutritional benefits for the host plant (Teste et al., 2020). In this context, as it concerns the black truffle producing systems, the research on diversity and dynamics of soil microbial communities together with the underlying ecological factors is essential to better understand their function in a specific ecosystem (Orgiazzi et al., 2012). In this sense, the species co-occurrence network analysis could contribute in deciphering the patterns of fungal community composition and understanding the complexity of black truffle dominated systems (Barberán et al., 2012). Two taxa present simultaneously at the same place are considered to co-occurring, and a set of taxa connected with links are usually depicted as networks (Goberna & Verdú, 2022). The analysis of the network composing of all co-occurring taxa has been considered a valuable approach to describe the microbial distribution across multiple sites (Goberna & Verdú, 2022; Prieto-Rubio et al.,

2024). On the other hand, the biodiversity and composition of biological communities is inherently associated with soil functioning (Wagg et al., 2021; Prieto-Rubio et al., 2024). Describing the fungal structure dynamics and identifying fungal co-occurrences or co-exclusions with *T. melanosporum* may give us important clues to understand the soil ecology of truffle-dominated brûlés.

Given the potential habitat effect associated with different land uses on soil microbial community structure (Piñuela et al., 2024) and functioning (Flores-Rentería et al., 2018), the study of co-occurring taxa in managed *vs* wild truffle producing environments could add valuable information on the precise microbial structure of these systems. More specifically, the variation of environmental conditions among these black truffle-producing systems may be linked to variations in functional fungal guilds that could play a relative important role in OM decomposition and nutrient´s mobilisation (Banerjee et al., 2016). This role, however, is often studied individually for specific guilds while ignoring others who live in the same environment (Fernandez & Kennedy, 2016). Studying soil carbon and nutrient cycling in relation to the whole fungal biodiversity is crucial for comprehending the nutritional feedbacks of the plant-soil system and for optimising management strategies, such as tailored fertilisation or pruning, of truffle-producing plantations.

Previously in **Chapter 2**, we showed that black truffle abundance impacted soil enzymatic activity in both natural and managed production systems, alongside other specific biotic and abiotic soil factors unique to each system. In **Chapter 3**, we aimed to go a step further and uncover the organisation of soil fungal communities in both black truffle-producing systems, and understand the role of *T. melanosporum* structuring them. Our specific objectives were 1) to examine how fungal richness (α-diversity) and species composition (β-diversity) varied between black truffle producing forests and plantations in two seasons: spring and autumn; 2) to study the fungal co-occurrence network structure in each truffle-producing system, and to analyse the role of *T. melanosporum* within it; and 3) to identify the main functional fungal guilds operating in soils of these truffle dominated systems, and their relative contribution to soil carbon and nutrient cycling.

Considering the less predictable environmental conditions of wild than managed ecosystems, we expected soil forest' fungal communities to be richer and more heterogeneous than those of plantations; a differential seasonal impact in soil fungal communities of the two truffle producing systems was also expected (hypothesis 1). Since microorganisms and plants have interacted over long evolutionary time in wild ecosystems (Song et al., 2019; Pérez-Lamarque et al., 2022), a more complex network structure was expected in black truffle producing forests than in plantations (hypothesis 2). In addition, given the strong allopathic black truffle effect inside the brûlé, we envisaged that *T. melanosporum* would markedly contribute to the soil fungal network structure acting as a keystone species within the community. Considering the distinct organic inputs from wild and agro-forestry

truffle systems (see **Table S3** in **Chapter 2**, and Barou et al., 2024), and the mycorrhizal-associated nutrient economy framework (Phillips et al., 2013), we anticipated finding different representative functional fungal guilds in each system. Specifically, we expected a greater prevalence of ectomycorrhizal *vs* saprotrophic fungi in forests compared to plantations, each differentially impacting the soil functioning (i.e., carbon and nutrient cycling) in these two systems (hypothesis 3).

To test these hypotheses, we investigated the soil fungal communities and other soil properties such as enzymatic activities related to carbon and nutrient cycling within the brûlé of black truffle producing holm oak forests and orchards, across the natural distribution area of this fungus in Spain.

2. Materials and Methods

2.1. Experimental design, soil sampling and processing

Eighteen sites along the natural distribution of the black truffle in Spain were selected. Per each site, two types of truffle-producing systems were considered: plantations (17 sites) and forests (10 sites). All plantations are effective truffle producing holm oak monocultures, more than 10-years-old, where standard agriculture management practices for truffle culture (mainly irrigation, tilling and pruning) are regularly conducted. In forests, *Quercus ilex* L. dominates the uppermost canopy layer. The study sites have continental to Mediterranean climate (see **Chapters 1** and **2** for more details of sites).

Four truffle-productive (according to local owners) *Q. ilex* trees were selected per site, and a total of 116 trees (68 in plantations and 48 in forests) were sampled in autumn 2019 and spring 2020. A metallic probe (4 cm diameter x 20 cm) was used to extract soil samples from the four orientations between the tree trunk and the limit of the brûlé, which were pooled into a unique composite soil sample per tree. Soil samples were stored at 4 °C until processing.

Physical-chemical soil analyses were performed as previously detailed in Barou et al. (2024) (see details in **Chapter 1**). The activity of seven soil enzymes related with the cycling of labile C (β-glucosidase and β-cellobiohydrolase), non-labile C (β-xylosidase and β-glucuronidase), N (chitinase and leucine) and P (alkaline phosphatase) was determined (see details in **Chapter 2**). Soil DNA was extracted and metabarcoding sequencing (Illumina MiSeq) was performed by targeting the fungal ITS1 region (see details in **Chapter 2**).

2.2. Bioinformatics analysis

Bioinformatics was conducted with the DADA2 pipeline (Callahan et al., 2016; R Core Team, 2022), as previously described in **Chapter 2**. Briefly, after primer's removal, sequences were filtered

and merged, taxonomically assigned, and clustered into OTUs (97% similarity), according to the v8.2 UNITE database. A final matrix of 8,654 fungal OTUs and 7,151,228 reads was obtained, after removing OTUs with less than five reads. Once the taxonomic assignations were obtained, OTUs closely related to a recognised fungal ecological guild (i.e., ectomycorrhizal, saprotrophs, pathogens, parasites, arbuscular mycorrhizal fungi, endophytes, epiphytes or lichens) were identified by the FUNGuild and FungalTraits databases (Tedersoo & Smith, 2013; Tedersoo et al., 2014; Nguyen et al., 2015; Põlme et al., 2020). Before any further analyses, rarefaction was applied to the whole dataset to check for suitable sequencing depth across all samples (**Fig. S1**), and the fungal database was normalised by dividing the number of sequences (i.e., reads) per OTU in a sample by the total number of sequences of that sample. The final dataset consisted of 231 samples since one sample was removed due to sequencing failure.

2.3. Co-occurrence fungal networks

To further investigate the structure of soil fungal communities, co-occurrence network analysis was performed following the methodology described by Prieto-Rubio et al. (2024). For this, the OTU's abundance matrix was first rescaled by the respective minimum sequencing depth for each dataset (i.e., whole dataset, and datasets separated by plantation or forest samples). Low frequent OTUs that appeared in <10 % of the samples in the respective datasets were discarded (Wagg et al., 2019). Then, to generate the fungal co-occurrence networks, the SPIEC-EASI algorithm was applied to each dataset with the *spiec.easi* function in the "SpiecEasi" R package (Kurtz et al., 2015). This method seeks to detect a link (i.e., co-occurrence) between any two nodes (i.e., two OTUs), assuming that their abundances are not independent and thus, discarding spurious associations between OTUs (Kurtz et al., 2015). The construction of networks was based on the Meinshausen & Bühlmann (2006) neighborhood selection method. The lambda ratio was set to 0.01 and, to detect the most parsimonious network, 50 values of lambda for every 100 cross-validation permutations were fitted by using the Stability Approach to Regularisation Selection (StARS) (Liu et al., 2010). The inference of the fungal networks and their preparation for visualisation were performed as described in Birt & Dennis (2021). The resulting fungal networks were graphically represented using the Gephi software v0.10 (Bastian et al., 2009), applying both the Fruchterman-Reingold distribution and ForceAtlas2 attraction-repulsion algorithms in the simulations (Fruchterman & Reingold, 1991; Jacomy et al., 2014).

To measure the contribution of each OTU – including that of *T. melanosporum* – in the network structure, network centrality metrics (degree, eigenvector, closeness, and betweenness centralities; Brede, 2012) and the hub score metric as proxy of keystone taxa (Deguchi et al., 2014), were calculated

in Gephi. In addition, the modularity algorithm proposed by Blondel et al. (2008) was also run in Gephi to determine the fungal network divisions into sub-communities, i.e., modules characterised by dense links inside the community compared to the sparser links with other communities (Newman, 2006). The nodes (equivalent to OTUs) belonging to the same module were assigned a distinctive colour, and the node size was determined by the hub score of that OTU. On the other hand, the links between the nodes were coloured according to the type of association, i.e., positive or negative. To unravel the black truffle interactions with the fungal community, the networks were finally filtered by the links established only with *T. melanosporum* and positive and negative co-occurrences with other fungi were identified.

2.4. Statistical analyses

In order to assess the effects of the type of truffle production system (hereafter "type") and different seasons of sampling (hereafter "season") on soil fungal diversity (hypothesis 1), a linear mixed model (LMM) was first run, with OTU richness (α-diversity) as response variable, type and season as fixed factors (including their interaction), and the site as random factor. The LMM was performed with *lmer* function from "lme4" R package (Bates et al., 2015) and pairwise comparisons to find significant differences between the different groups were conducted with *lsmeans* (Lenth, 2016). β-diversity of soil fungal communities was then calculated based on the Bray-Curtis OTU's dissimilarity matrix, and nonmetric multidimensional scaling (NMDS) was used to draw the compositional differences within the respective type and season treatments, with the *metaMDS* function from "vegan" R package (Oksanen et al., 2022). Statistical significance of factors was determined by permutational multivariate analysis of variance (PERMANOVA, permutations = 9999) with the *Adonis* function using the same Bray-Curtis OTU's dissimilarity matrix. In addition, the vectors of pH, electric conductivity (EC), organic matter (OM), active carbonate, and N, P, K, Mg and Fe content were also fitted in the NMDS with the *envfit* function.

To investigate the impact of the factors type and season on the co-occurrence soil fungal network (hypothesis 2), we first calculated different structural network properties i.e., OTU richness, the number of links between OTUs, the network complexity (ratio of links to OTU richness), and the detrended network complexity (by discarding the OTU richness intrinsic variation, see in Wagg et al., 2019) using the total dataset (*N* = 231 samples). The structural network properties were used as response variables in LMMs build with the same fix (type, season) and random (site) factors as described above.

Then, co-occurrence networks were separately built for plantations (*N* = 135) and forests (*N* = 96) datasets. To evaluate the contribution of *T. melanosporum* in the fungal network structure and

identify other potential keystone taxa, besides each OTU hub-score, a comparative ranking list among OTUs was calculated (adapted from Brandon-Mong et al., 2020). Briefly, each OTU was first assigned to a rank based on the values of each of its network centrality metrics (degree, eigenvector, closeness, betweenness); then, the rankings of all metrics were summed for each OTU, resulting in a final total rank assigned to each OTU. The top-10 highly ranked OTUs were considered as keystone taxa, and the position of *T. melanosporum* within this list was used to evaluate its contribution to the soil fungal network structure.

To test the hypothesis 3, the effect of factors type and season on the most representative ecological fungal guilds (ectomycorrhizal, saprotroph, pathogen/parasite, *T. melanosporum* and unknown fungal guild) was first analysed by LMMs, with the abundance of each guild as response variable and the model syntaxes similar to that previously explained. Then, to evaluate if soil carbon and nutrient cycling was explained by the respective fungal guilds, LMMs were fitted by testing the response of enzymatic activities representative of these cycles (i.e., dependent variables) to the relative abundance and richness of each fungal guild. In order to select the best LMM, the *dredge* function of "MuMIn" R package (Bartoń, 2015) was applied to perform random combinations of all potential explanatory variables (i.e., fungal guild's abundance or richness) initially included in the models. Final models included the combination of those predictors that gave the best model fit according to the Akaike' Information Criterion (Akaike, 1974). The site was included as random factor in all models and separated models were run for each subset of enzymes respectively involved in soil labile-C (β-glucosidase and β-cellobiohydrolase), non-labile C (β-xylosidase and β-glucuronidase), N (chitinase and leucine) and P (alkaline phosphatase) cycles.

The graphical representation of results (i.e., α-diversity, abundances of ecological fungal guilds and most abundant fungal taxa) was performed with the "microeco" R package (Liu et al., 2021). All analyses were performed with the R software version 4.2.1 (R Core Team, 2022).

3. Results

3.1. Diversity of soil fungal communities

A total of 8,654 fungal OTUs were identified, with an average of 325 ± 77 (SD) OTUs per sample. The 38.2 % of all OTUs (*N* = 3,309) – that were also the most abundant – were shared in plantations and forests, with close to 30 % of OTUs (low abundant) uniquely found in each respective type of truffle producing system (**Fig. 1a**). Approximately half of OTUs (representing most of the abundance) were found at both seasons, and 21-25 % of them were only found either in autumn or spring, but their relative abundance represented just 1-2 % of the total (**Fig. 1a**).

Contrary to that expected (hypothesis 1), no significant differences in fungal α-diversity were observed between truffle producing plantations and forests (p = 0.142), and only marginal differences due to season were observed (p = 0.072) (**Fig. 1b**). No interaction among factors was detected. However, when β-diversity was analysed, significantly dissimilar fungal communities were found within the treatments of the respective factors studied: type (F = 13.05, $p < 0.001$, R^2 = 0.053) and season (F = 3.8, $p < 0.001$, R^2 = 0.016). As expected, fungal species composition was more homogeneous in plantations than in forests, and in autumn than in spring (**Fig. 1c**). β-diversity of plantations appeared correlated with pH, and the content of K and active carbonates, while in forests it was mainly correlated with soil OM, EC, and N, Fe and Mg contents (**Fig. 1c**). In terms of seasonal patterns, the fungal community community likely correlated with EC in autumn and N and active carbonates in spring (**Fig. 1c**).

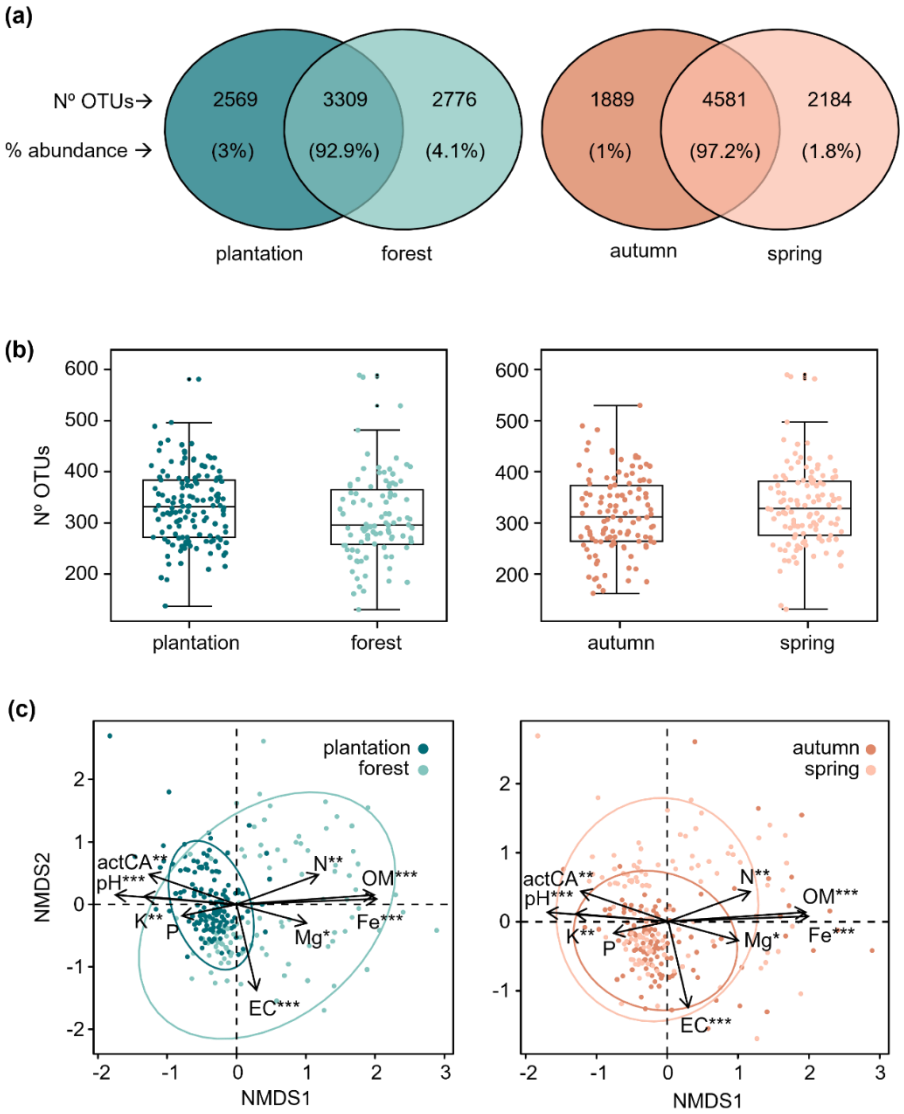


Figure 1 Sequencing yields (a), α-diversity (b) and β-diversity (c) of soil fungal communities, depending on the factors studied: type of black truffle producing system (plantation, forest) and season of sampling (autumn, spring) (*N* samples = 231; *N* OTUs = 8,654). In (b) medians, min, max and quartiles are represented. In (c) β-diversity is represented by nonmetric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities for soil fungal community data: ellipses indicate 95% confidence intervals of fit and vectors represent the strength and direction of soil variables weight (k = 2, stress = 0.22, R^2 = 0.95). EC = electric conductivity, OM = organic matter, actCA = active carbonates.

Across the soil samples from the two truffle-producing systems and seasons of sampling, the most abundant phylum was Ascomycota, representing 64 – 73 % of the total fungal abundance, followed by Basidiomycota and Mortierellomycota, with 15 – 26 % and 5 – 8 % relative abundance, respectively (**Fig. 2a**). *Tuber melanosporum* represented between 13 – 22 % of total Ascomycota abundance, depending on the treatment. In addition, Among the assigned fungal genera in the total OTU's dataset (71 % of OTUs), the top-ten most representative included *Tuber*, as the most abundant one in both types of truffle-producing systems and both seasons, followed by *Mortierella*, *Solicoccozyma* and *Fusarium* in plantations and *Mortierella*, *Tomentella, Inocybe* and *Picoa in* forests (**Fig. 2b**).

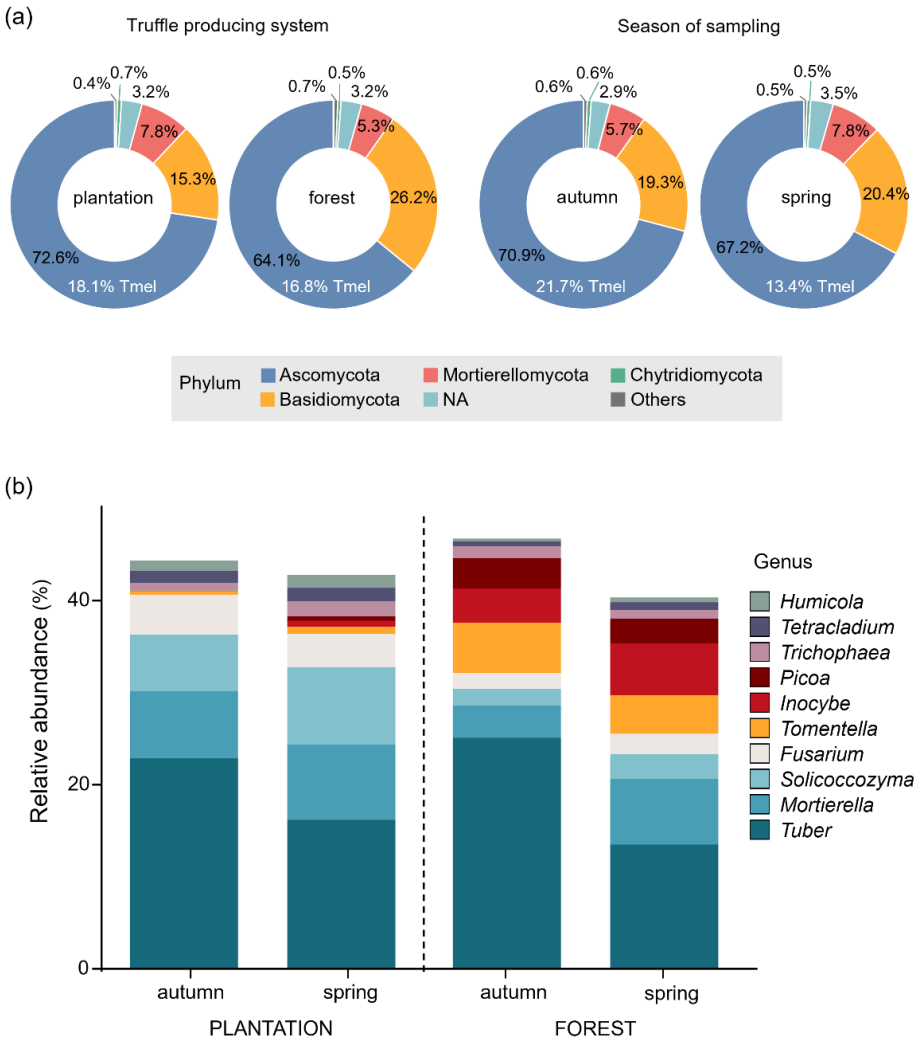


Figure 2 (a) Main fungal phyla in each black truffle producing system and season studied. Percentages represent the mean relative abundance of each phylum and the proportion of *Tuber melanosporum* (Tmel) abundance. NA are not taxonomically identified OTUs. (b) Top-ten most abundant genera across treatments of the studied factors: type of truffle-producing system and seasons of sampling (*N* = 231, OTUs = 8,654).

3.2. Fungal network structure and T. melanosporum *representativeness*

After removing low frequent OTUs (see 2.4 in the Materials and Methods section), total fungal network (*N* = 231 samples) was composed of 706 co-occurring OTUs that were present in, at least, 10 % of the samples and showed 4,512 links among OTUs. The type of truffle producing system did significantly affect the structural network properties and, contrary to that expected (hypothesis 2), more complex fungal network and more links between taxa were observed in plantations than in forests, while no effect of season was observed (**Fig. 3**, **Fig. S2**, **Table S1**).

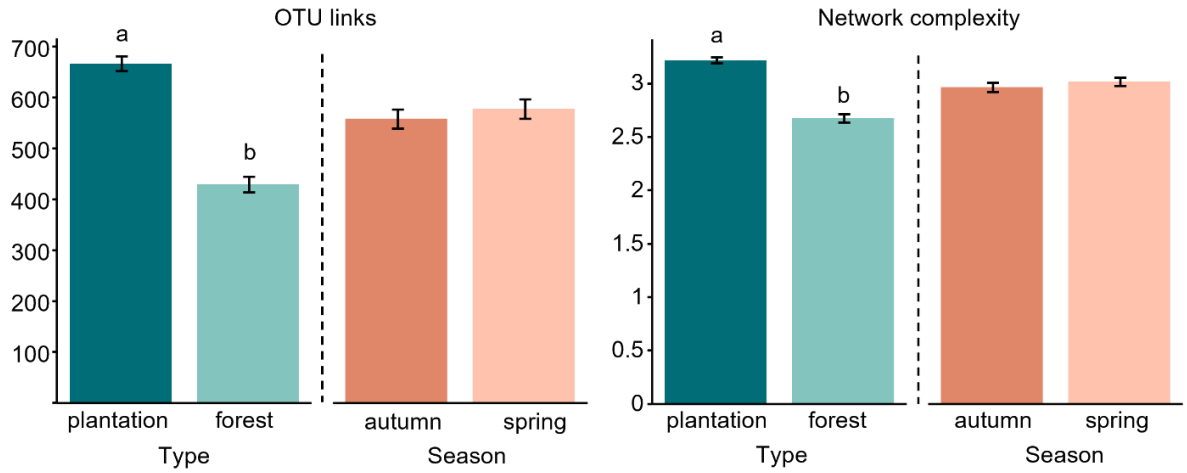


Figure 3 Structural properties of soil fungal networks in two types of black truffle producing systems (plantation, forest) and two seasons of sampling (autumn, spring). Bars represent the mean ± SE of each network property (*N* samples = 231; OTUs = 706, present in >10% of samples) and letters indicate significant differences between the treatments of a given factor, derived from linear mixed models and pairwise comparisons (see **Table S1**). OTU links = number of co-occurrences between OTUs; Network complexity = ratio between the number of links and OTU richness.

Regarding the node centrality metrics, the top-10 taxa with the highest total ranking in all network metrics (degree, eigenvalue, closeness and betweenness) included eight Ascomycota genera and/or species (*Stachybotrys chartarum, Setophaeosphaeria badalingensis, Arthrinium* sp., *Chaetosphaeronema* sp., *Penicillium jensenii, Humicola fuscoatra, Botryotrichum atrogriseum, Microdochium novae-zelandiae*), one unclassified Ascomycota OTU, and one Mortierellomycota OTU (*Mortierella* sp.). *Tuber melanosporum* was in the 163rd position (out of 706), i.e., within the first 23 % OTUs of this ranking, and dropped to 37 % (261st position) when only the hub score (proxy of keystone taxa) was considered. These results indicated that, contrary to what we expected (hypothesis 2), *T. melanosporum* was not acting as a keystone species structuring the total co-occurring fungal network inside the brûlé.

To investigate more closely the representativeness of *T. melanosporum* in each type of truffle producing system, separate network analyses were further conducted for plantations and forests. The

resulting fungal networks were composed of 728 and 641 co-occurring OTUs in plantations and forests respectively, which were distributed across 13 different modules in both cases (**Fig. 4**). Each module was composed of at least 25 OTUs and some modules were more central than others (i.e., modules with higher centrality metrics: more relevant and connected within the network), both in plantations (e.g., modules 3 and 6; PERMANOVA, p = 0.003, R^2 = 0.02) and in forests (e.g., modules 0, 3 and 8; PERMANOVA, p = 0.001, R^2 = 0.02) (**Fig. 4**). As regards of *T. melanosporum* centrality, its position in the network metrics' ranking was 276th in plantations and the 58th in forests, i.e., within the first 38 % and 9% network influential OTUs respectively while this position shifted to 35.8 % and 21.2 % respectively when only the hub score was taken into account. Given that, although in neither truffle-producing system it could be characterised as keystone species, *T. melanosporum* likely had a more central role in forests than in plantations.

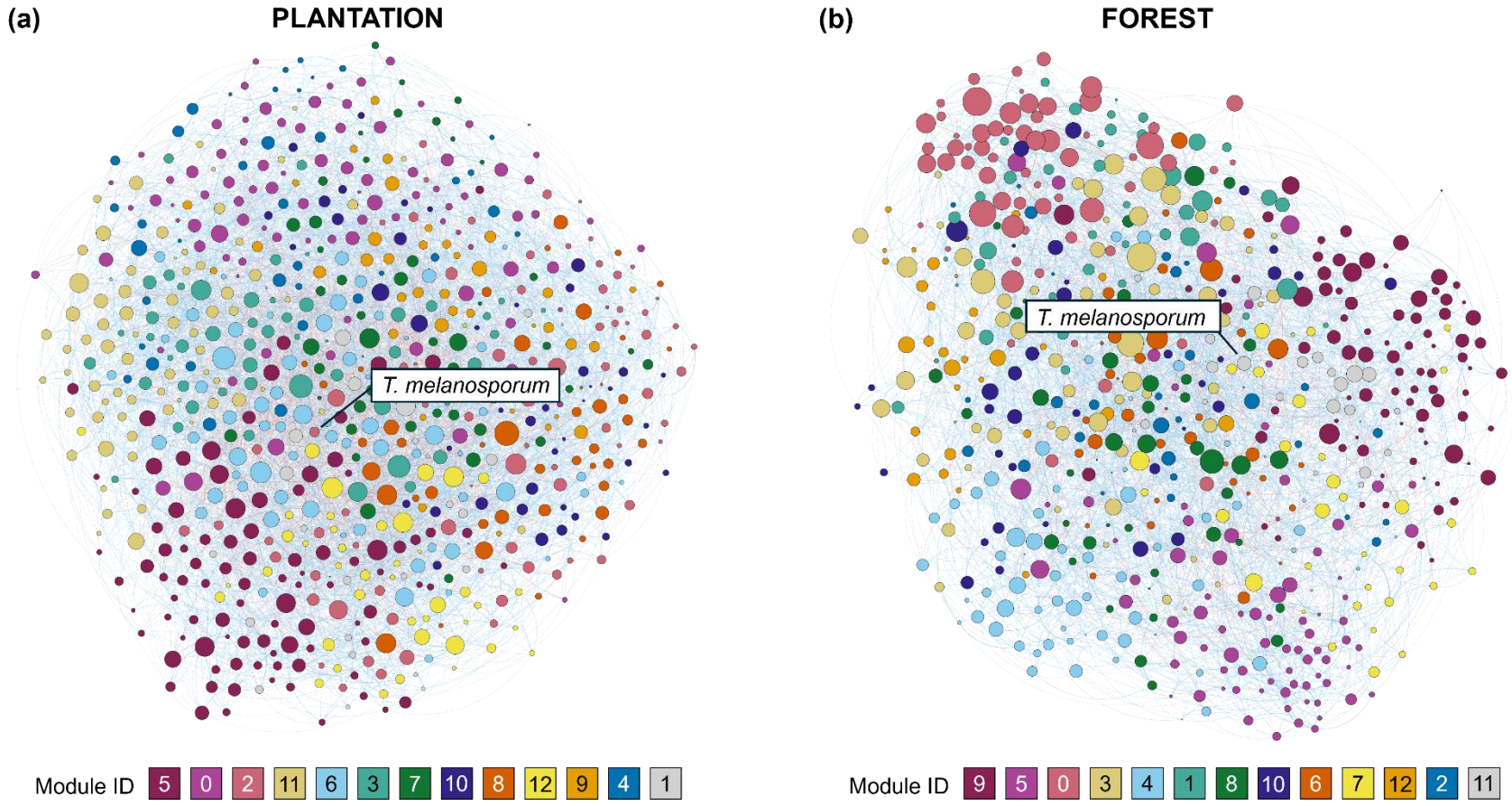


Figure 4 Co-occurrence soil fungal networks found in black truffle producing plantations (a) and forests (b). The node size is related to the hub score index (i.e., a proxy of keystone taxa), and the node colour indicates a separate module inferred by Gephi (different colours were assigned to module IDs for each system). Blue links represent co-occurrences and orange links represent co-exclusions between nodes (i.e., OTUs).

Tuber melanosporum was connected either positively (co-occurrence) or negatively (co-exclusion) with 2.3 % and 2.8 % of the total fungal OTUs in plantations and forests, respectively (**Fig. 5**). One third of these connections in plantations and more than half in forests represented co-occurrences of *T. melanosporum* with other fungi, while the rest of links represented co-exclusions (**Table S2**). Among these links, *Trichothecium crotocinigenum* (Schol-Schwarz) Summerb. was the only OTU co-occurring with *T. melanosporum* in both systems (**Fig. 5**, **Table S2**). Different *Mortierella* OTUs showed links with *T. melanosporum* in the co-occurring networks, both in plantations (negative link), and in forests (positive link). Particularly in plantations, *T. melanosporum* showed negative links with saprotrophic OTUs, both positive and negative links with OTUs characterised as pathogens-parasites and a positive link with one AMF OTU (**Fig. 5a**, **Table S2**). On the other hand, in forests, *T. melanosporum* was linked either positively or negatively with saprotrophic, pathogens-parasites and ECM OTUs (**Fig. 5b**, **Table S2**).

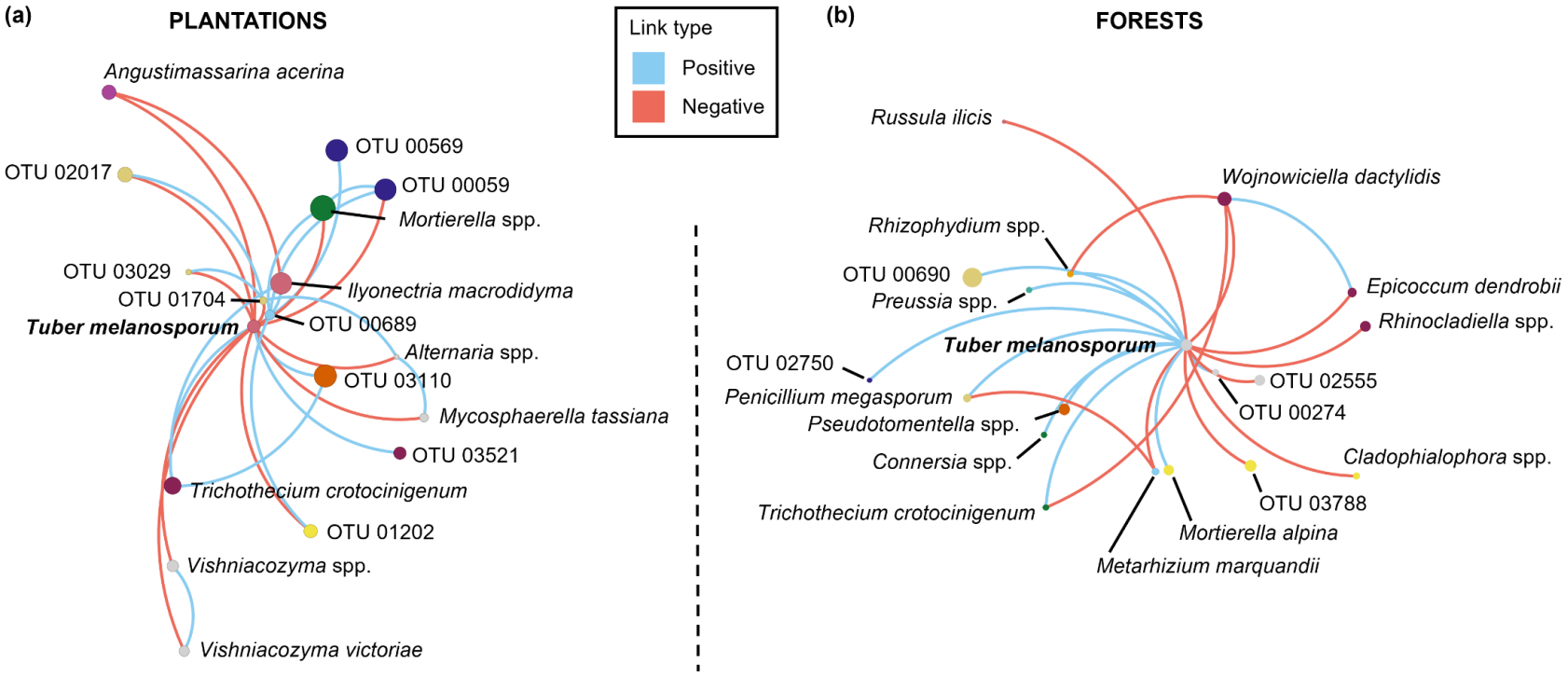


Figure 5 Co-occurrences (positive links) and co-exclusions (negative links) between *Tuber melanosporum* and other fungal species in black truffle productive plantations (a) and forests (b).

3.3. Fungal guilds and soil functioning

Approximately 35 % of fungal taxa (*N* = 3,000 OTUs) were assigned to an ecological guild, representing 71 % of the total fungal abundance in the whole database. In plantations, the relative abundance of saprotrophic, pathogens/parasites and *T. melanosporum* was significantly higher than in forests (**Fig. 6**, **Table S3**). As expected (hypothesis 3), forests showed greater abundance of ectomycorrhizal OTUs (other than *T. melanosporum*) than plantations. Differences in fungal guilds abundance were also found between seasons regarding the saprotrophic OTUs that were significantly more abundant in spring than in autumn, while the opposite was observed for *T. melanosporum* (**Fig. 6**, **Table S3**).

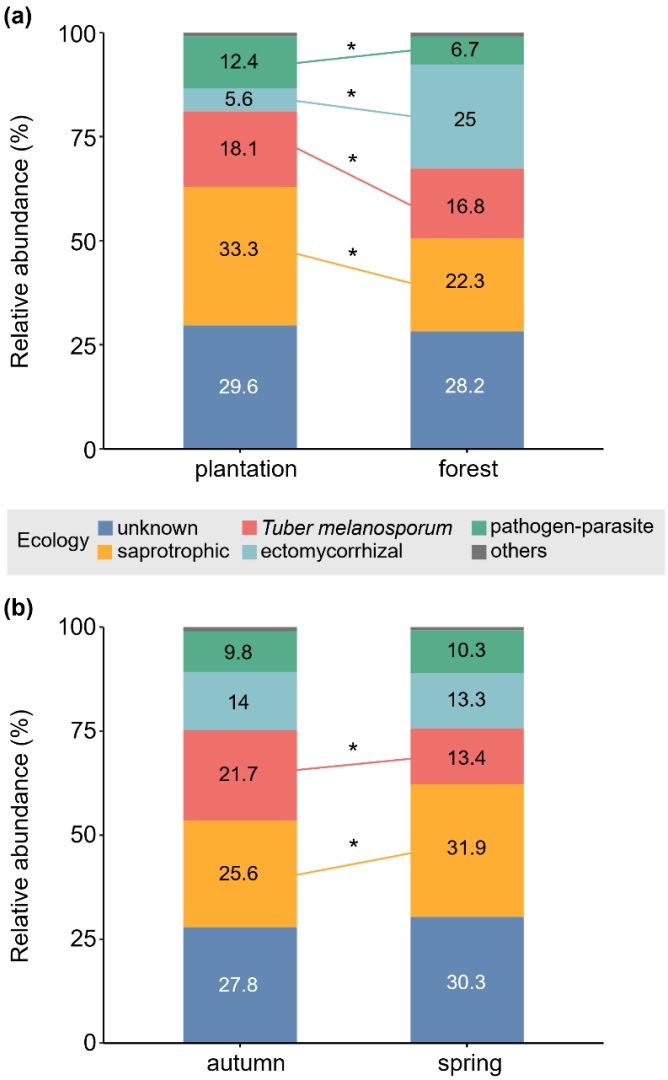


Figure 6 Relative abundance of the main fungal guilds, together with *Tuber melanosporum*, found in each truffle producing system and sampling season. For a given fungal guild, asterisk represents significant differences between treatments per factor (a) type or (b) season, derived from linear mixed models (see **Table S1**). "Others" includes fungi classed as arbuscular mycorrhizal, endophytes, epiphytes and lichens.

To test if soil fungal guilds could explain soil carbon and nutrient cycling, linear mixed models were performed with all guilds and separately for each type of truffle producing system (**Table 1**).

Table 1 Linear mixed models for (a) plantations and (b) forests, separately built for each C (labile: β-glucosidase, β-cellobiohydrolase; non-labile: β-xylosidase, β-glucuronidase), N (chitinase, leucine) or P (alkaline phosphatase) cycle as dependent variables and the relative abundance or richness of different soil fungal guilds as predictors (*N* = 231 samples). The site was set as random factor in all models. R^2m = marginal variance explained by predictors; R^2c = conditional variance explained by predictors and random factor. Significant *p*-values (< 0.05) are in bold.

(a) PLANTATIONS

Variable	Predictor	Estimate ± SE	*t*-value	*p*-value	R^2m	R^2c
	Fungal guild's abundance					
Labile C-cycle	Ectomycorrhizal	0.013 ± 0.008	1.598	0.112	0.021	0.404
Non-labile C-cycle	*(Intercept)*	-	-	-	-	0.289
N-cycle	Pathogens/Parasites	-0.017 ± 0.009	-1.924	0.057	0.045	0.298
	Saprotrophs	0.007 ± 0.004	1.972	0.051		
P-cycle	*(Intercept)*	-	-	-	-	0.537
	Fungal guild's richness					
Labile C-cycle	Saprotrophs	-0.013 ± 0.007	-1.749	0.083	0.019	0.405
Non-labile C-cycle	Saprotrophs	-0.003 ± 0.002	-1.814	0.072	0.024	0.278
N-cycle	Saprotrophs	-0.008 ± 0.003	-3.104	**0.002**	0.066	0.326
P-cycle	*(Intercept)*	-	-	-	-	0.537

(b) FORESTS

Variable	Predictor	Estimate ± SE	*t*-value	*p*-value	R^2m	R^2c
	Fungal guilds abundance					
Labile C-cycle	*(Intercept)*	-	-	-	-	0.279
Non-labile C-cycle	Ectomycorrhizal	-0.002 ± 0.001	-1.505	0.136	0.025	0.231
N-cycle	*(Intercept)*	-	-	-	-	0.384
P-cycle	*(Intercept)*	-	-	-	-	0.203
	Fungal guilds richness					
Labile C-cycle	*(Intercept)*	-	-	-	-	0.279
Non-labile C-cycle	*(Intercept)*	-	-	-	-	0.201
N-cycle	Saprotrophs	-0.005 ± 0.003	-1.563	0.121	0.024	0.365
P-cycle	*(Intercept)*	-	-	-	-	0.203

In plantations, saprotrophs were predictors (marginal positive effect of relative abundance and significant negative effect of richness) of soil N cycling (**Table 1a**), as well as the abundance of pathogen-parasites (marginal negative effects). In forests, none of the fungal guilds could explain significantly the enzymatic activity (**Table 1b**).

4. Discussion

4.1. Diversity of soil fungal communities in wild and managed truffle producing ecosystems

In this study we assessed the fungal community composition of black truffle brûlés in two types of truffle-producing systems and two sampling seasons. Contrary to our first hypothesis, soil fungal richness was similar in plantation and forests, and in autumn and spring. Although it is usual that soil fungal richness decreases when land use intensity increases, the opposite pattern or even unclear changes have also been observed in an extensive literature review (Balami et al., 2020). A study in *Quercus*-dominated habitats found a strong association of fungal richness with tree species richness (Saitta et al., 2018), and this could explain the similarity of fungal richness in our sampled sites where *Q. ilex* was the dominant tree species. However, in our regional-scale study, soil fungal β-diversity did show significant differences between the type of truffle producing system or the seasons. A recent study of soil microbial diversity across Europe has identified land-use perturbation, climate, soil properties and vegetation cover as the main influencing factors on soil fungal diversity (Labouyrie et al., 2023). Our results agree with the differences in β-diversity of soil fungal community previously observed in Mediterranean and temperate climate sites, sampled across four seasons (Piñuela et al., 2024), although in this case no seasonal effects were detected probably due to the limited number of plots compared to our study.

Despite the spatial and seasonal differences in our studied sites, soil fungal communities were principally composed of Ascomycota, in agreement with previous studies (Fu et al., 2016; Herrero de Aza et al., 2022), while some of the most abundant genera apart from *Tuber* were *Mortierella*, *Solicoccozyma*, *Fusarium* and *Tomentella*, similarly as detected in other studies (De Miguel et al., 2016; Herrero de Aza et al. 2022).

4.2. Soil fungal networks in black truffle-dominated systems

In our study, the fungal co-occurrence network across all sampled black truffle brûlés showed a strong dependency on the type of production system rather than the sampling season, which is consistent with the results of Piñuela et al. (2024). Network links and complexity were higher in plantations than in forests, which may indicate a more stable fungal community in the former than the latter system (Griffiths & Philippot, 2013). However, contradictory results regarding the fungal network complexity have been observed and, for example, the conversion of a tropical rainforest to rubber plantation increased the fungal network complexity (Lan et al., 2022), while the opposite occurred with the conversion of a deciduous forest to a coniferous plantation (Nakayama et al., 2019).

Different ecological processes can potentially contribute to changes in co-occurrence patterns of communities at the regional scale, including habitat filtering, competition or neutral processes (Horner-Devine et al., 2007; Maaß et al., 2014). Differences in soil fungal network structure among forest and plantations have been, however, regularly explained by the variation in soil properties (Wang et al., 2024), or different herbaceous cover, and subsequently in rhizosphere conditions (Nakayama et al., 2019; Tang et al., 2024) (i.e., habitat filtering). Further studies are needed to disentangle the main ecological processes regulating the biotic (and abiotic) interactions in truffle-dominated ecosystems.

Once *T. melanosporum* is well established within the brûlé, its allelopathic effect likely enables it to dominate by serving as a strong environmental filter for plants and soil microbial communities (Napoli et al., 2010; Streiblová et al., 2012). In fact, in **Chapter 2**, we showed that *T. melanosporum* abundance negatively correlated with that of other fungi in wild and managed truffle-producing systems, and also with the overall fungal richness in plantations. But surprisingly, despite its dominance, neither in the whole soil fungal network nor in those separated by plantations and forests, *T. melanosporum* appeared as keystone species and rather saprotrophs and pathogens-parasites had a central role in the fungal network of black truffle brûlés. In fact, in both truffle-producing systems, only a very small proportion of taxa of the total fungal community interacted directly with *T. melanosporum*. Among the most abundant genera, only *Mortierella* OTUs were related with *T. melanosporum* in the co-occurring networks, both in plantations (negative link), and in forests (positive link). This genus of saprotrophic fungi has been reported to collaborate with AMF in phosphorus acquisition (Osorio & Habte, 2001; Tamayo-Velez & Osorio, 2017). In addition, the only common OTU linked with *T. melanosporum* in both systems was *Trichothecium crotocinigenum*. This fungus was assigned as plant pathogen (primary life-style) and litter saprotroph (secondary life-style) according to fungal trait databases, but literature has also described it as a potato-associated endophytic fungus with antipathogenic effects (Yang et al., 2018, 2020). Further research is needed to understand the positive association of *T. melanosporum* with this fungal species, as well as the interactions with other fungi within the brûlé.

4.3. Functional contribution of soil fungal guilds

In terms of seasonal dynamics, and independently of the type of black truffle producing system, higher abundance of saprotrophs was found in spring than autumn, probably related with soil temperature (Piazza et al., 2019), and/or the increased carbon flux from host roots to the soil. Concerning wild and managed truffle-producing systems, significant differences were observed for most soil fungal guilds. The abundance of saprotrophs and pathogens-parasites was significantly

higher in plantations than forests, while the opposite happened for ECM fungi. Since in plantations the host trees were initially inoculated with *T. melanosporum*, this fungus probably had advantage over other ECM species to colonise new root-tips of the host trees (Kennedy & Bruns, 2005). Additionally, soil legacies can play a role since plantations are established in previous agricultural lands usually dominated by saprotrophs, and devoid of ECM propagules compared to forests (Phillips et al., 2013). It has been also reported that intensive land use shapes soil microbial communities and increases the abundance of potential plant-pathogens (Idbella & Bonanomi, 2023). In any case, the extent to which the dominant effect of *T. melanosporum* is synergistically linked to the dynamics of saprotrophs and/or pathogen-parasitic fungi in plantations, as well as ECM in forests, and the potential functional consequences of these interactions, still requires further investigation.

When fungal guilds were further tested for their contribution in soil functioning, certain saprotrophs (i.e., marginal positive effect of relative abundance, but significant negative effect of richness) and potential pathogens-parasites significantly predicted soil nitrogen cycling, but only in plantations not in forests. A recent study in Mediterranean oak stands showed that saprotrophs were the main fungi responsible for high C and N mineralisation (Adamo et al., 2022); this can be probably related to fast decomposition of high-quality litter by saprotrophs, releasing nutrients in the mineral soil that can be mined by mycorrhizal species (Phillips et al., 2013). It cannot be ruled out that other soil saprotrophs as bacteria can be facilitating N cycling in soil (Fernandez & Kennedy, 2016; Lebreton et al., 2021). Regarding forests sites, neither the relative abundance nor the richness of the main fungal guilds could explain the soil enzymatic activity, which was mainly driven by spatial-temporal dynamics and specific soil properties, as previously seen in **Chapter 2**, or by other biotic factors not studied in the present work.

In all cases, the site accounted for a considerable amount of variance of carbon and nutrient cycling in relation to fungal guilds in models, implying that local site-specific conditions play a crucial role in nutrient cycling. However, our study demonstrates that certain common functional patterns may be discernible at a broader regional scale.

5. Conclusions

In this study, we aimed to define the structure of soil fungal communities associated with black truffle producing plantations and forests, and to decipher the relationship between fungal ecological guilds and carbon and nutrient cycling in soils dominated by *T. melanosporum*. This is the first study exploring the co-occurrence fungal network structure in black truffle brûlés and identifying fungal links with *T. melanosporum*. The soil fungal communities were more homogeneous (i.e., more similar taxa) and showed a more complex network structure in plantations than in forests, indicating more stable

fungal communities, probably related to the uniform environmental conditions and reduced plant community diversity in monoculture plantations. *T. melanosporum* had a higher central role in forests than in plantations, but in neither system was a keystone species, while it showed a few direct fungal links, mainly with saprotrophic and pathogens-parasites taxa. The ecological fungal guilds explained partially the carbon and nutrient cycling in plantations, particularly saprotrophs and parasites-pathogens. Understanding the ecology, including the biodiversity and functioning, of black truffle-dominated soils will provide a stronger foundation for making informed decisions regarding the management of black truffle plantations.

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7. Supplementary material

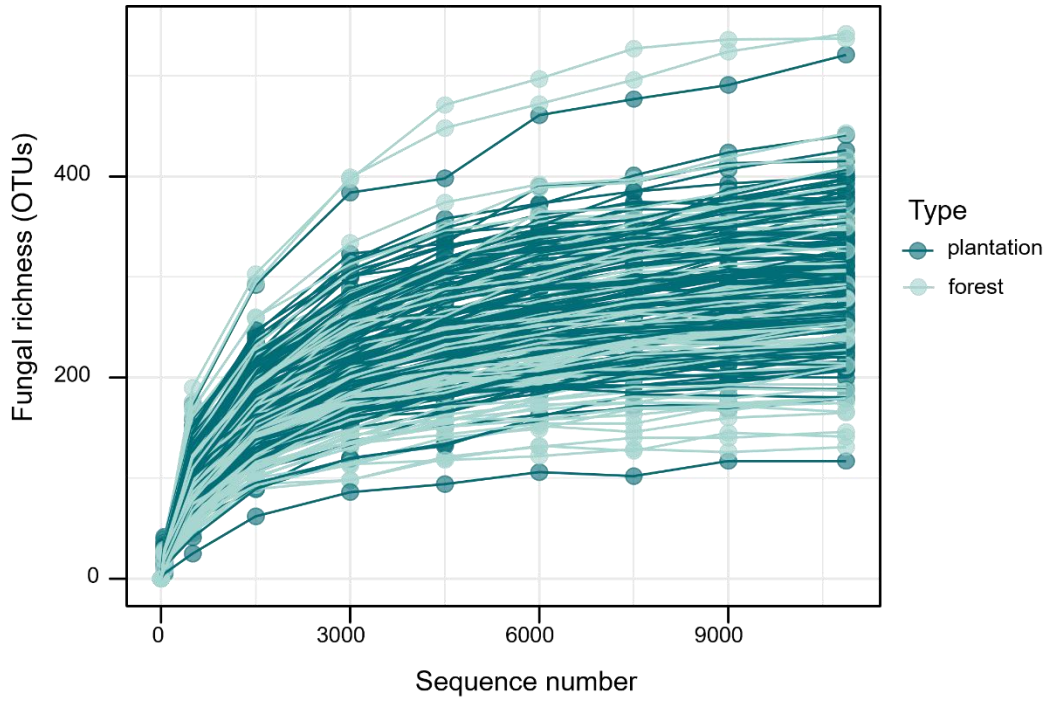


Figure S1 Rarefaction curves of all samples (*N* = 231) in each type of black truffle producing system (plantation and forest). The graph was performed with "microeco" R package.

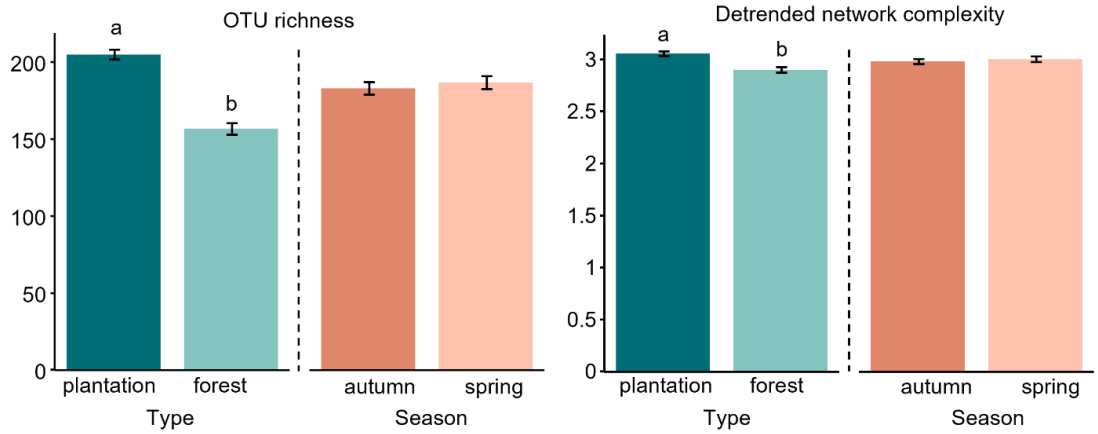


Figure S2 Structural properties of the co-occurrence fungal network in brûlés of different black truffle producing systems (plantations, forests) and seasons of sampling (autumn, spring). Bars represent the mean ± SE of each network property (*N* samples = 231; *N* OTUs = 706) and letters represent significant differences between the respective factor treatments, derived from linear mixed models (**Table S2**). Detrended network complexity = complexity after discarding the intrinsic variation of OTU richness.

Table S1 Linear mixed models fitted for structural properties of the co-occurrence fungal network in black truffle brûlés (see **Fig. 3** and **Fig. S2**). Type of truffle producing system and season of sampling were included as fixed factors and site as random factor in models. Significant *p*-values (< 0.05) are shown in bold. R^2m = marginal; variance explained by fixed factors; R^2c = conditional; variance explained by fixed and random factors. Network complexity = ratio of links to OTU richness. Detrended network complexity = complexity after discarding the intrinsic variation of OTU richness.

Structural network property	Predictor	Estimate ± SE	F value	*p*-value	R^2m	R^2c
OTU richness	type	41.431 ± 5.316	60.743	**< 0.001**	0.23	0.423
	season	3.782 ± 4.287	0.779	0.379		
Fungal links	type	211.054 ± 23.6	79.974	**< 0.001**	0.286	0.445
	season	20.277 ± 19.193	1.116	0.292		
Network complexity	type	0.496 ± 0.051	96.382	**< 0.001**	0.321	0.495
	season	0.057 ± 0.041	1.937	0.165		
Detrended network complexity	type	0.161 ± 0.032	25.016	**< 0.001**	0.081	0.559
	season	0.027 ± 0.025	1.195	0.276		

Table S2 Taxonomic identification from Phylum to Species and fungal guild assignment of OTUs co-occurring with *Tuber melanosporum* Vittad. in truffle producing plantations and forests (see **Fig. 5**). Positive links represent co-occurrence and negative links represent co-exclusion with *T. melanosporum*. ECM = ectomycorrhizal fungi; SAP = saprotrophic; PAT-PAR = pathogen-parasites; AMF = arbuscular mycorrhizal fungi; UN = unassigned fungal guild.

PLANTATIONS								
OTU ID	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Fungal guild**	**Type of link**
OTU00021	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Ilyonectria	*I. macrodidyma*	PAT-PAR	positive
OTU00050	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	Mycosphaerella	*M. tassiana*	PAT-PAR	negative
OTU00059	--	--	--	--	--	--	UN	negative
OTU00357	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	--	SAP	negative
OTU00419	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	--	PAT-PAR	negative
OTU00492	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	Trichothecium	*T. crotocinigenum*	PAT-PAR	positive
OTU00569	Ascomycota	Sordariomycetes	Coniochaetales	--	--	--	UN	positive
OTU00636	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	Vishniacozyma	*V. victoriae*	SAP	negative
OTU00689	--	--	--	--	--	--	UN	negative
OTU01199	Ascomycota	Dothideomycetes	Pleosporales	Amorosiaceae	Angustimassarina	*A. acerina*	PAT-PAR	negative
OTU01202	--	--	--	--	--	--	UN	negative
OTU01203	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	Vishniacozyma	--	SAP	negative
OTU01704	--	--	--	--	--	--	UN	negative
OTU02017	--	--	--	--	--	--	UN	negative
OTU03029	--	--	--	--	--	--	UN	negative
OTU03110	Ascomycota	Sordariomycetes	--	--	--	--	UN	positive
OTU03521	Glomeromycota	Glomeromycetes	Diversisporales	Diversisporaceae	--	--	AMF	positive

FORESTS								
OTU ID	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**	**Fungal guild**	**Type of link**
OTU00004	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	Mortierella	*M. alpina*	SAP	positive
OTU00028	Basidiomycota	Agaricomycetes	Russulales	Russulaceae	Russula	*R. ilicis*	ECM	negative
OTU00070	Ascomycota	Dothideomycetes	Pleosporales	Sporormiaceae	Preussia	--	SAP	positive
OTU00184	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	Epicoccum	*E. dendrobii*	PAT-PAR	negative
OTU00274	Ascomycota	--	--	--	--	--	UN	positive
OTU00492	Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	Trichothecium	*T. crotocinigenum*	PAT-PAR	positive
OTU00508	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Rhinocladiella	--	SAP	negative
OTU00690	--	--	--	--	--	--	UN	positive
OTU00717	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	Wojnowiciella	*W. dactylidis*	SAP	negative
OTU00718	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Metarhizium	*M. marquandii*	PAT-PAR	negative
OTU00798	Basidiomycota	Agaricomycetes	Thelephorales	Thelephoraceae	Pseudotomentella	--	ECM	positive
OTU01198	Chytridiomycota	Rhizophydiomycetes	Rhizophydiales	Rhizophydiaceae	Rhizophydium	--	PAT-PAR	positive
OTU01717	Ascomycota	Eurotiomycetes	Chaetothyriales	Herpotrichiellaceae	Cladophialophora	--	SAP	negative
OTU02555	--	--	--	--	--	--	UN	negative
OTU02586	Ascomycota	Leotiomycetes	Thelebolales	Pseudeurotiaceae	Connersia	--	SAP	positive
OTU02750	Chytridiomycota	Spizellomycetes	Spizellomycetales	Powellomycetaceae	--	--	SAP	positive
OTU03788	--	--	--	--	--	--	UN	negative
OTU04160	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*P. megasporum*	SAP	positive

Table S3 Linear mixed models fitted for the relative abundance of *Tuber melanosporum* and that of the most abundant ecological fungal guilds in each soil sample (see **Fig. 6**). Type of producing system and season of sampling were included as fixed factors and site as random factor in models. Interactions among fixed factors were not significant and thus were discarded from the models. Significant *p*-values (< 0.05) are shown in bold. R^2m = marginal variance explained by fixed factors; R^2c = conditional variance explained by fixed and random factors.

Variable	Predictor	Estimate ± SE	F value	*p*-value	R^2m	R^2c
T. melanosporum	type	0.704 ± 0.176	16.079	**<0.001**	0.163	0.452
	season	-0.996 ± 0.147	45.928	**<0.001**		
Ectomycorrhizal	type	-1.405 ± 0.163	74.098	**<0.001**	0.246	0.441
	season	-0.134 ± 0.138	0.939	0.334		
Saprotrophs	type	0.514 ± 0.062	69.060	**<0.001**	0.269	0.449
	season	0.233 ± 0.053	19.622	**<0.001**		
Pathogen-parasite	type	0.937 ± 0.107	75.928	**<0.001**	0.217	0.536
	season	0.074 ± 0.089	0.690	0.407		

Chapter 4

Aromatic plants and their associated arbuscular mycorrhizal fungi outcompete *Tuber melanosporum* in compatibility assays with truffle-oaks

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Simple summary

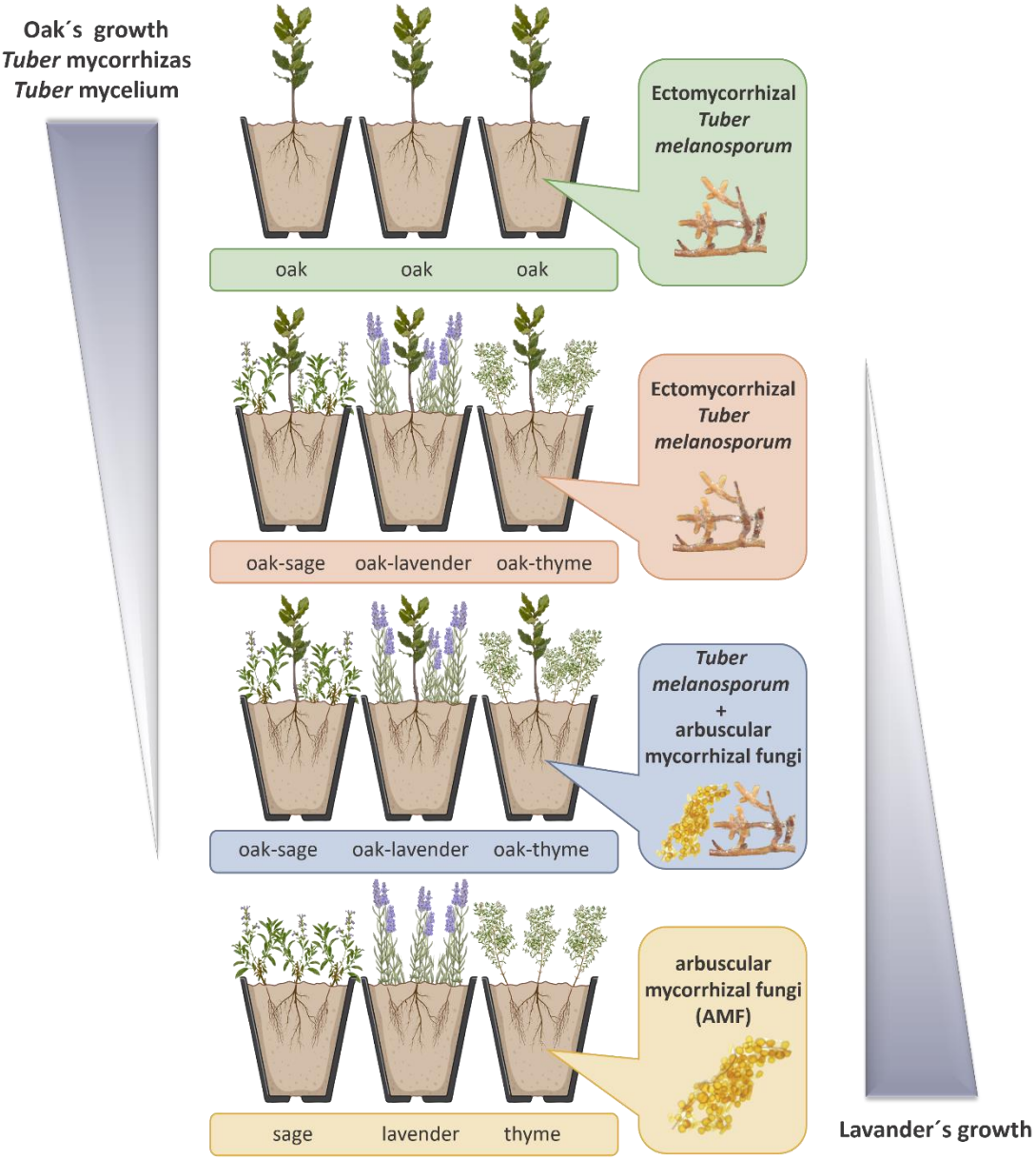
Truffle culture is a fairly profitable agricultural practice, yet there is a long waiting period to reach peak sporocarp production from the point when ectomycorrhizal truffle-oak seedlings are planted in the field. Adding a secondary crop, such as medicinal and aromatic plants, could enhance the sustainability of truffle agro-forest systems. In this work, we study the relationships between oaks and aromatic plants and their associated mycorrhizal fungi (either ectomycorrhizal or arbuscular mycorrhizal, respectively) under controlled conditions. A reciprocal competition effect is revealed between oaks and aromatic plant species, as well as between the different types of mycorrhizal fungi. Our results indicate that managing arbuscular mycorrhizal fungi in truffle plantations is a relevant factor to be considered when establishing the dual cultures of plant species and mycorrhizal types in intercropping systems.

Abstract

The high value of black truffle recompenses the slow growth of the fungus when established in the field. Adding a secondary crop, such as medicinal and aromatic plants (MAPs), could further enhance the sustainability of truffle production agro-forest systems. The dual cultures of ectomycorrhizal truffle-oak seedlings and MAPs (lavender, thyme, and sage) previously inoculated and non-inoculated with native arbuscular mycorrhizal fungi (AMF), were established to evaluate plant-fungi relationships. After 12 months in a shadehouse, plants' growth, mycorrhizal colonisation, and extraradical soil mycelium (both of *Tuber melanosporum* and AMF) were measured. Overall, truffle-oaks' growth was negatively affected by the presence of MAPs, especially when inoculated with AMF. In turn, the presence of truffle-oaks barely affected the co-cultured MAPs, and only lavenders showed a significant growth reduction. All AMF-inoculated MAPs showed higher shoot and root biomass than non-inoculated ones. Compared to truffle-oaks growing alone, the presence of co-cultured MAPs, especially when they were AMF-inoculated, significantly decreased both the ectomycorrhizas and soil mycelium of *T. melanosporum*. These results reveal the strong competition between AMF and *T. melanosporum* and warn about the need for the protection of intercropping plants and their associated symbiotic fungi to avoid reciprocal counterproductive effects in mixed truffle-oak-AMF-MAP plantations.

Keywords: *Tuber melanosporum*; truffle-oaks; aromatic plants; arbuscular fungi; mycorrhizas; intercropping; competition; lavender; thyme; sage

Graphical abstract



1. Introduction

Truffles are edible ectomycorrhizal fungi greatly appreciated and widely used in international haute cuisine (Reyna & Garcia-Barreda, 2014). Like other truffle species, the black truffle (*Tuber melanosporum* Vittad.) forms mycorrhizal associations with the roots of diverse hosts, such as the Quercus species, to complete its lifecycle and produce edible fruitbodies (Iotti et al., 2016). During the twentieth century, the production of the black truffle moved from woodlands to planted truffle orchards (Murat, 2015), playing an economic, cultural, and structural role in Mediterranean landscapes (Blondel, 2006). Truffle production in truffle orchards emerges a minimum of five years after the trees are planted, and it takes from seven to eleven years to achieve peak production (Reyna & Garcia-Barreda, 2014). Thus, intercropping strategies, i.e., the agronomic practices of growing two or more compatible crops simultaneously on the same field, could allow truffle growers to integrate a secondary crop in pre-productive truffle plantations to improve the ecosystem services and profitability. Compared to monocultures, diverse agroecosystems created by intercropping generate remarkable benefits, such as increased biodiversity, enhanced pest control, and erosion prevention, as well as diversified economic yields (Rosa-schleich et al., 2019). Moreover, this traditional farming system enhances not only crop productivity but also the efficient utilization of resources, both above- and below-ground (Brooker et al., 2015; Stomph et al., 2020). Intercrops should be designed to take maximum advantage of their potential for complementarity and the facilitation of the involved crops, considering the choice of species or variety, input levels, sowing dates, and spatial-temporal configuration (Costanzo & Bàrberi, 2014).

Intercropping with medicinal and aromatic plants (MAPs) can increase the stability of agroforest ecosystems (Tripathi et al., 2019). For example, intercropping with basil (*Ocimum basilicum* L.) and summer savory (*Satureja hortensis* L.) significantly increased soil organic nitrogen and available nitrogen contents, improving soil quality in orchard ecosystems (X. Chen et al., 2014). A recent survey in Southern France indicated that truffle growers relate intercropping with MAPs to the improvement of truffle production and soil organic matter and, to the enhanced drought resilience of truffle orchards (Geoffroy et al., 2018; Martin-Chave, 2019). Among compatible crops, MAPs are a suitable option to combine with Mediterranean truffle plantations, since they are adapted to the same ecological conditions as truffle-oaks. However, truffle brûlés, the areas with scarce vegetation due to the allelopathic effects of truffle mycelium in the soil, could limit intercropping possibilities by reducing plant growth and associated soil microbiota (Streiblová et al., 2012).

It is necessary to understand the ecological processes underlying intercropping designs, such as the nature of the relationships between plants and mycorrhizal fungi, to reach an outcome that

increases sustainability and ecosystem services (Brooker et al., 2015). Interactions between plants and microbes benefit plants by increasing the acquisition of nutrients, producing growth hormones (Das et al., 2022), and defending against diseases and pests (Berendsen et al., 2012). More specifically, in MAPs, previous studies have shown an increased yield of shoot biomass and essential oil when sage (*Salvia officinalis* L.), thyme (*Thymus vulgaris* L.), and oregano (*Origanum vulgare* L.) plants were inoculated with arbuscular mycorrhizal fungi (AMF) (Tarraf et al., 2015). In the case of coriander (*Coriander sativum* L.), the AMF inoculation served as a biofertilizer, promoting the growth and nutrient acquisition of plants (Oliveira et al., 2016).

Few studies have shown the effects of interactions between truffle plants (forming ectomycorrhizas, ECM) and shrubs and herbs (companion plants, mostly forming arbuscular mycorrhizas, AM) either cultivated or growing spontaneously in truffle brûlés (González-Armada et al., 2010; Taschen et al., 2020). Previous studies on AM-ECM fungal interactions were mostly conducted at the scale of plant individuals hosting multiple types of mycorrhizal fungi (Y. L. Chen et al., 2000; Queralt et al., 2019). Among them, plants of the genus Quercus are confirmed to form both kinds of mycorrhizal symbioses, forming AM and ECM structures (Teste et al., 2020). However, the simultaneous reciprocal interaction of plants hosting AM and ECM fungi has not yet been sufficiently explored (Netherway et al., 2021). Knoblochová et al. (2017) studied the co-occurrence of the AM grass *Calamagrostis epigejos* Roth (L.) and the predominantly ECM tree *Salix caprea* L. at post-mining sites that were spontaneously colonized by vegetation. Aside from the microenvironmental effects on both fungal communities, the presence of *S. caprea* significantly decreased AM fungal abundance in soil as well as AMF colonization and richness in *C. epigejos* roots. McHugh and Gehring (2006) observed the opposite trends in semi-arid, pine-juniper woodland, where the density of the AM shrub *Juniperus monosperma* (Engelm.) Sarg. was negatively correlated with the ECM colonization of *Pinus edulis* Engelm. and ECM fungal abundance in soil (Haskins et al., 2004).

More recently, Taschen et al. (2020) showed that a selection of companion plants, all of them hosting AMF, promoted the development of truffle mycelium in a rhizotron experiment established with evergreen oaks (*Quercus ilex* L.), growing together with different species of AM plants. Glomeromycotina (AMF) mycelia in soils inoculated with *T. melanosporum* was six times lower than in non-inoculated rhizotrons. However, the experimental AMF inoculation of companion plants was not included in this assay and, consequently, not controlled. In our study, we designed a mesocosm experiment (Odum, 1984) to evaluate the interactions between truffle-inoculated evergreen oak seedlings and three species of MAPs, namely lavender (*Lavandula officinalis* Chaix.), thyme, and sage, either non-inoculated or inoculated with native AMF. We hypothesize that: (i) the growth of co-cultured truffle-oaks and MAPs will be reduced as compared to monocultures; (ii) in the case of MAPs,

this negative effect of co-culture will be reduced by mycorrhization with native AMF; and (iii) *T. melanosporum* colonization (ectomycorrhizas and extra-radical mycelium) will outcompete AMF in dual cultures.

2. Materials and Methods

2.1. Experimental setup, plant material, and AM inoculum production

A mesocosm experiment was conducted during a period of one year in a shadehouse with open ends located at the Institute of Agrifood Research and Technology (IRTA) in Cabrils, Barcelona (41°30'58.6" N, 2°22'36.7" E). The area has a Mediterranean climate with a mean annual rainfall of 614 mm and a mean annual temperature of 16 °C.

One-year-old evergreen oaks inoculated with spores of *T. melanosporum* were obtained in June 2020 from a commercial nursery ('Viveros Alto Palancia', Castellón, Spain). The seed provenance was 'Sistema Ibérico', lot 12/1049_OR/2019/A001/00002. The seedlings were originally produced in 650 cc containers filled with a commercial substrate containing peat, perlite, and disinfected soil. Ten seedlings were randomly selected for visual examination under a stereomicroscope for truffle ectomycorrhizal colonization before establishing the experiment. The initial percentage of ectomycorrhizas (number of ECM tips/total number of, at least, 200 short roots) was around 20.6 ± 9.3 (SD). Plantlets of lavender, thyme, and sage produced in peat plugs were obtained from a commercial nursery (Aldrofeu and Associats, Riudarenes, Girona, Spain) in April 2020.

Native AM inoculum was isolated from different weeds growing inside the brûlés of three productive truffle plantations in Spain: Batea (Tarragona), La Matilla (Segovia), and Montan de Tost (Lleida). Different AM plant species were carefully collected in June 2019 from the brûlés. The rhizosferic soil attached to the plants' roots from each site was added into 250 cc pots filled with sterilized sand (120 °C, 1 atm, 1 h) from a quarry. A trap plant (*Allium porrum* L.) was then transplanted into each pot to recover the AM inoculum as described in Camprubí and Calvet, (1996). The final AM inoculum consisted of a mixture of colonized roots, spores, and extraradical mycelium in a matrix of sand. The inocula from the three sites were examined under the microscope to identify the presence of AMF propagules, and all of them displayed Glomus-type spores (**Figure S1a**). The three AM inocula were mixed in the same volume, and half of the MAPs were inoculated with 10 mL of AMF mixed inoculum in 250 cc individual pots filled with the same sand, while the other half were transplanted but remained non-inoculated. In July 2020, the MAPs were assessed for AM root colonization using the gridline intersection method (Giovannetti & Mosse, 1980) and were then ready for transplantation.

2.2. Experimental design and management

In June 2020, topsoil up to 30 cm depth was collected in a field next to a productive *T. melanosporum* plantation, free of potential host plants, in Batea (Tarragona, Spain). The soil was mechanically mixed with 10 % perlite, distributed into 10 L plastic bags, and autoclaved (120 °C, 1 atm, 1 h). The soil was analyzed after sterilization (**Table S1**). The physico-chemical soil analysis showed normal or non-limiting parameters for a truffle producing area (Colinas et al., 2007) with a low organic matter and phosphorus and high calcium content. Sixty-four containers (25 cm diameter, 8 L capacity each) were filled with the autoclaved soil-perlite mixture. One truffle-oak seedling was transplanted into each container and three MAPs (either sage, lavender, or thyme), either mycorrhized with AMF or not, were transplanted around each oak next to the edge of the container to study dual plant and fungal interactions (**Figure 1**). Control monoculture treatments with either AM-MAPs or truffle-oak seedlings growing alone were also included in the design.

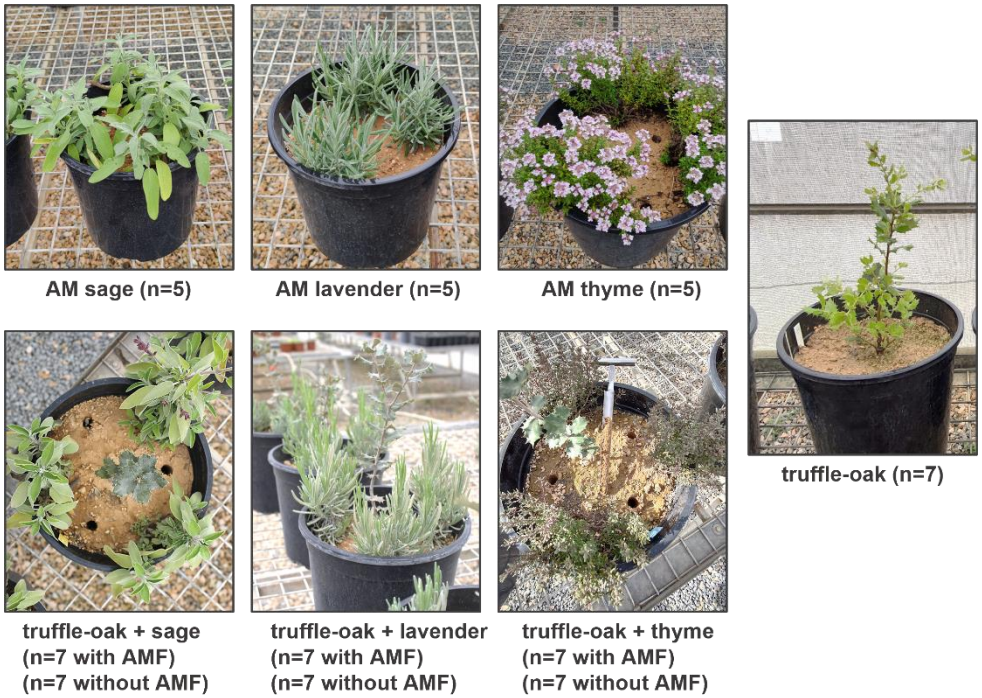


Figure 1 Experimental set-up of interactions between evergreen oak ectomycorrhizal with *Tuber melanosporum* (truffle-oak) and three species of medicinal and aromatic plants (MAPs), inoculated with arbuscular mycorrhizal fungi (AMF) or non-inoculated. Truffle-oak and MAPs growing alone were used as controls.

The experiment was established in July 2020 and maintained under regular watering in the shadehouse for 12 months. No fertilizers were applied throughout the experiment. The positions of the containers on the bench were rotated once a month. In the middle of the experiment (December 2020), MAPs were partially pruned to avoid physical competition with oaks.

2.3. Plants growth and physiological parameters

At the end of the experiment, in July 2021, we measured the height of all plants and the diameter of the oaks, as well as counting the number of spikes of lavenders. We also measured the leaf mass per area (LMA) and the chlorophyll content of new leaves with a SPAD-502, (Konika Minolta Sensing, Inc., Tokyo, Japan). Plants were then harvested and root and shoot biomass were measured after air-drying at 65 °C for 48 h in a drying oven (UF1060, Memmert GmbH & Co. KG, Schwabach, Germany). The dry weight of MAPs was averaged for each container after adding the dry weight of the middle-season pruning.

2.4. Quantification of mycorrhizas

The percentage of the ECM of T. melanosporum in oak plants at the end of the experiment [(No. mycorrhizas/No. of total short roots) × 100] was calculated from a minimum of 200 short roots of each plant examined under a stereomicroscope. A sample of roots of the MAPs was clarified and stained (Koske & Gemma, 1989) and the percentage of cortex infected by the AMF was estimated visually under a stereomicroscope, as described in Giovannetti and Mosse (1980).

2.5. Molecular analyses of soil samples

Soil cores were taken at three points, equidistant between the MAPs and the center of each container, by using a 15 cm-long cheese probe (**Figure 1**) and then pooled into one composite sample per container. Soil samples were sieved with a 2 mm mesh and dried at 50 °C in a heater with recirculating air. Then, we extracted total DNA from 0.25 g of each soil sample using the DNeasy Power Soil HTP 96 kit (Qiagen, Hilden, Germany). The concentration of the yielded DNA per sample was determined with a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Wilmington, DE, USA). The extracted DNA was stored at −20 °C until further analysis.

The extraradical mycelium biomass of *T. melanosporum* from each soil sample was quantified by real-time Taqman® PCR (qPCR) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems™ by Thermo Fisher Scientific, Wilmington, DE, USA), as described in Parladé et al. (2013). The standard curve was created using DNA extracted from known gleba amounts of a fresh immature sporocarp (Parladé et al., 2007). Five ten-fold DNA dilutions were prepared to generate a standard curve from 40 to 0.0004 mg mycelium/g soil.

The soil biomass of AMF in the containers with MAPs and truffle-oaks was quantified by comparative qPCR-CT ($\Delta\Delta C_T$ method) using the same DNA extractions as described above. This method was originally designed to calculate the relative expression ratio of a target gene in different samples

(Livak & Schmittgen, 2001), using reference genes as internal reaction control to normalize mRNA levels between different samples. In our experiment, we used this method with the total soil DNA extractions to calculate the relative quantity between AM fungal mycelium in soils with AM-MAPs growing alone (controls) and soil samples from co-cultures (aromatic plants and oaks). The fold changes of AM fungal mycelium in the soil samples were calculated with the $2^{-\Delta\Delta Ct}$ formula ($\Delta C_T = C_T$ target gene − C_T reference gene; $\Delta\Delta C_T = \Delta C_T$ target sample − ΔC_T reference sample). The primers AM1 (Helgason et al., 1998) and AMG1F (Hewins et al., 2015) were used to quantify Glomeromycota, as described in Bodenhausen et al. (2021), with the same thermocycling conditions except for the annealing temperature, which was set at 60 °C. To normalize the samples to the same amount of total soil fungi, several genes were tested as endogenous controls (**Table S2**). We chose ITS1F (Gardes & Bruns, 1993) and ITS2 (White et al., 1990) primers, covering the ITS1 rDNA region of most fungi (Mello et al., 2011), because they were the only primers giving consistent amplification in all the soil samples. The relative amount of AMF mycelium and the total amount of fungi present in the samples was calculated separately for each plant species (lavender, sage, or thyme).

To check the possible bias from using the multicopy ITS1 rDNA region as endogenous gene, we performed qPCR analyses from the DNA extractions of different soil samples, corresponding to different inoculation treatments, and adjusted accordingly in order to obtain the same amount of DNA (measured with a Qubit fluorometer). We obtained very close amplification curves from all the samples (**Figure S2**), indicating that the same fungal DNA concentrations from different samples showed similar C_T values.

2.6. Statistical analyses

The data of plant growth, plant physiology, mycorrhizal colonization, and soil mycelial biomass were analyzed through analysis of variance (ANOVA), after checking for the normality and variance homogeneity of data. The shoot biomass of truffle-oaks was analyzed by ANCOVA to remove the influence of the initial height of the plants. When necessary, variables were log or log(x + 1) transformed to fit normal distribution. The percentages of *T. melanosporum* mycorrhizas were analyzed through generalized linear models considering the binary distribution of colonized vs. non-colonized roots. Soil mycelium colonization relationship between *T. melanosporum* and AMF was assessed by Spearman's correlation analysis.

All analyses were performed with the R software v. 4.2.1 (R Core Team, 2022), and figures with statistical data were produced using the "plotly" package (Sievert, 2020).

3. Results

3.1. Truffle-oaks and MAPs development

Overall, truffle-oak growth (shoot dry weight, height, diameter) and LMA were negatively affected by the presence of MAPs, particularly when these were inoculated with AMF (**Figure 2A**; **Table S3**). Root dry weight and chlorophyll content in oak leaves were unaffected by co-cultured MAPs (**Table S3**). Only in co-cultures did the two-way ANOVA analysis of truffle-oak shoot biomass, considering the initial height of the oaks as a covariate, show a significant effect of AMF inoculation on MAPs (F = 28.340, $p < 0.001$), of MAP species identity (F = 4.766, $p = 0.015$), and the interaction of the two (F = 3.994, $p = 0.0274$).

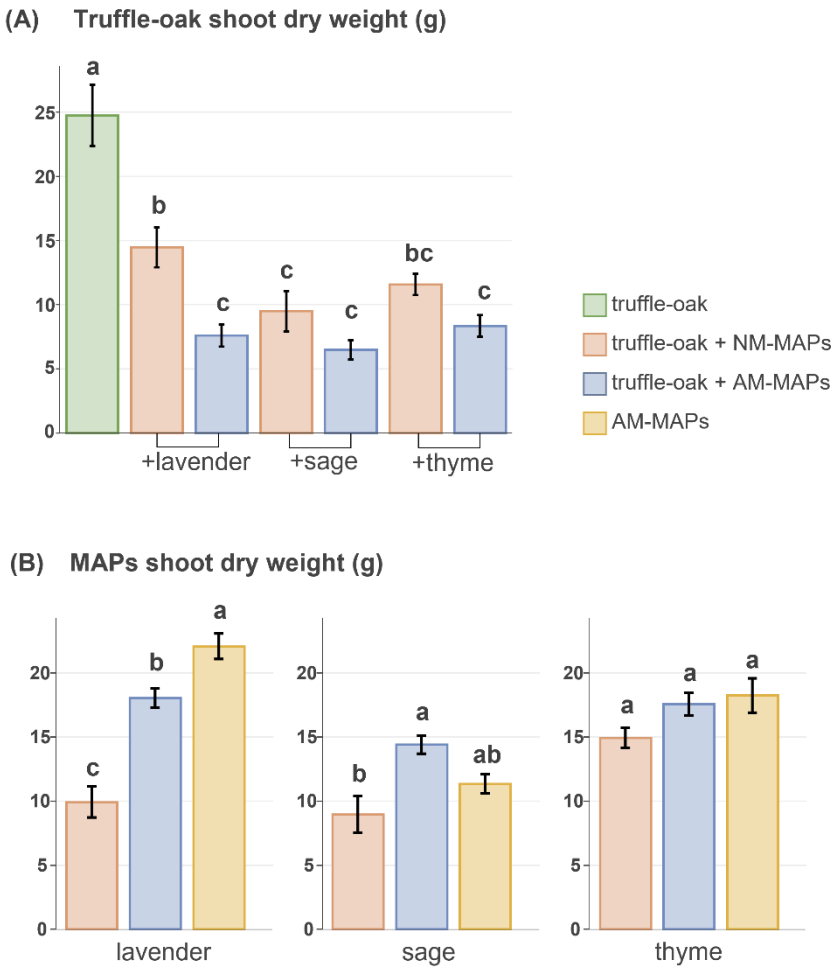


Figure 2 (A) Shoot dry weight of truffle-oak seedlings growing alone ($n = 7$, green) or in the presence of MAPs, either non-inoculated (NM, $n = 7$ per species, pink) or inoculated with AMF (AM, $n = 7$ per species, blue). (B) Shoot dry weight of lavender, sage, and thyme co-cultured with truffle-oaks and either non-inoculated (NM, pink) or inoculated with AMF (AM, blue), and growing alone ($n = 5$ per species, yellow). Data include means ± standard errors. Significant differences between means were analyzed by ANOVA for each MAP species separately and by ANCOVA in the case of truffle-oaks with the initial height as covariate. Treatments sharing the same lowercase letter are not significantly different (Tukey's test, $p < 0.05$).

The presence of truffle-oaks affected MAPs differently (**Figure 2B**; **Table S4**). Only AM lavenders growing together with truffle-oaks had significantly less biomass than those growing alone. All the inoculated MAPs in a co-culture with truffle-oaks had significantly more shoot biomass than non-inoculated ones. The number of lavender spikes was significantly lower in non-inoculated plants growing together with truffle-oaks as compared to those inoculated with AM growing either in monoculture or co-culture with oaks (**Table S4**).

3.2. Quantification of Tuber melanosporum

Truffle-oaks growing alone showed, on average, 44% ± 22.7 (SD) of *T. melanosporum* ectomycorrhizas (ECM) at the end of the experiment. This represents more than twice the initial percentage of ectomycorrhizas, indicating that the established mesocosms conditions were appropriate to allow the secondary colonization of the oak roots. The amount of extraradical mycelium and ectomycorrhizas of *T. melanosporum* drastically decreased in the presence of MAPs, especially of AM-inoculated MAPs (**Figure 3**). The percentage of *T. melanosporum* ECM was highly correlated to the soil mycelium biomass (Spearman, r = 0.7056, $p < 0.0001$). Both *T. melanosporum* mycorrhizas and extraradical mycelium were significantly correlated with truffle-oak biomass (Spearman, r = 0.6692 and r = 0.7555, respectively, $p < 0.001$).

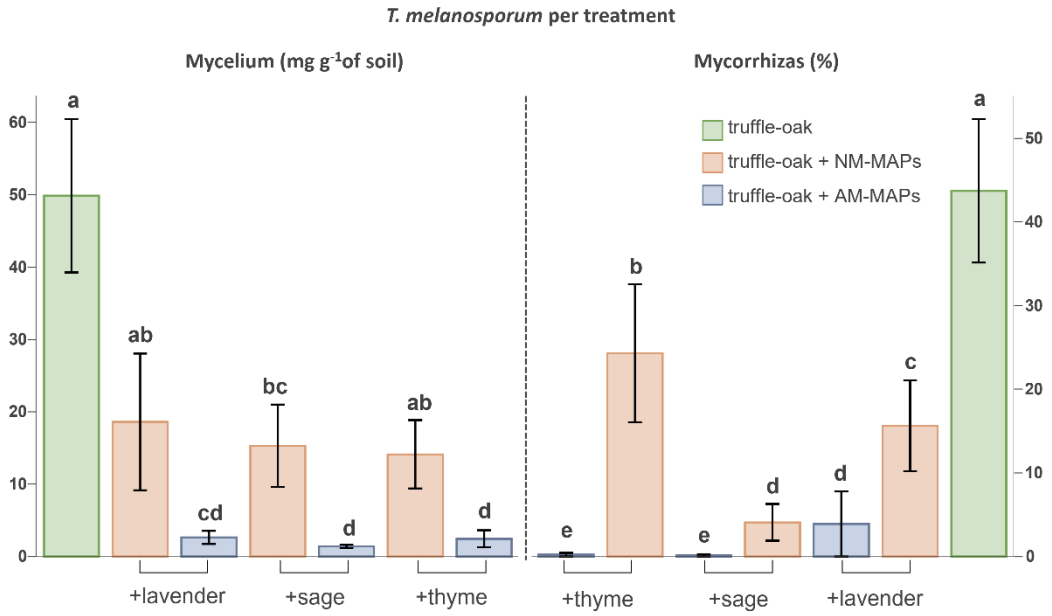


Figure 3 *Tuber melanosporum* extraradical mycelium biomass (graph on the left) and truffle ectomycorrhizal percentages of oaks (graph on the right). Truffle-oaks were growing alone (n = 7) or in presence of MAPs, either non-inoculated (NM, n = 7 per species) or inoculated (AM, n = 7 per species) with native AMF. Data include means and standard errors. Mycelium biomass was analyzed by one-way ANOVA ($F_{(6,42)}$ = 12.65, p < 0.0001) and the percentages of ectomycorrhizas by generalised linear models (Chi-Square$_{(6,42)}$ = 1466, p < 0.0001) assuming a binomial distribution of colonised vs. non-colonised roots. In each graph, treatments sharing the same lowercase letter are not significantly different (Tukey's test, p < 0.05).

3.3. AMF quantification in MAPs and truffle-oaks

The relative quantification of AM fungal mycelium in soil revealed a significant effect of the presence of truffle-oaks on AMF. The AMF mycelium in soils with co-cultures of truffle-oaks and AM-MAPs was expressed by as much as 35–40% of their respective control treatments consisting of monocultures of each AM-MAP (**Table S5**). No significant differences in AMF mycelium reduction were found between co-cultured MAP species' identities (ANOVA, $F_{(2,18)}$ = 0.0393, p = 0.9616). The relative quantity of AMF mycelium in soil with the co-cultures of truffle-oak and AM-MAPs (i.e., the fold change relative to control AM-MAPs growing alone) was negatively correlated with the *T. melanosporum* mycelium biomass (Spearman, r = −0.5922, p = 0.0055), indicating a significant competition between extraradical mycelium of both types of mycorrhizal fungi. Conversely, the percentage of AMF root colonization was not significantly reduced in co-cultured MAPs, as compared to MAPs growing alone (**Table S6**).

Truffle-oak seedlings growing together with AM-MAPs showed an elevated colonization by AMF (~80%) (**Figure S3**). Since no AM colonization was observed on oaks growing alone, the presence of AM colonization was derived, exclusively, from the companion AM-MAPs. No significant correlation

was found between the percentage of AM mycorrhizas and truffle-oak biomass (Spearman, r = −0.1233, p = 0.5929).

4. Discussion

The plantation of medicinal and aromatic plants in truffle orchards is considered a convenient alternative to overcome the lack of benefits of early truffle plantations until sporocarp onset (Geoffroy et al., 2018). MAPs and oak trees establish different kinds of mycorrhizal associations, arbuscular mycorrhizas and ectomycorrhizas, respectively. On the other hand, truffle mycelium exerts a strong allelopathic effect that usually limits the growth of both plants and microorganisms in its influential area (the brûlé) (Streiblová et al., 2012). This study was designed to evaluate the competition relationships between truffle mycelium (establishing ectomycorrhizas with oaks) and companion MAPs and their associated AMF under controlled conditions.

The co-culture of young truffle-oaks and MAPs resulted in the growth reduction of truffle-oaks (especially the shoot dry weight) and the loss of ectomycorrhizas as compared to the control, and this effect was strengthened when the companion MAPs were inoculated with native AMF, supporting our first and second hypotheses. Notably, the essential oils of many MAPs contain volatile compounds with strong antifungal activity, especially against Ascomycetes (Abu-Darwish et al., 2013; Martins et al., 2015; Erland et al., 2016; Lavin et al., 2016; Mandras et al., 2016; Palla et al., 2020). Although this effect has not yet been tested on *Tuber* mycelium in the field, in vitro experiments have shown that these compounds inhibit its growth (Leonardi et al., 2021).

The growth reduction of the oaks could be explained by the competition for soil nutrients and the overall loss of *T. melanosporum* ectomycorrhizas. However, the effects of the ectomycorrhizas of *T. melanosporum* on the growth of *Quercus* seedlings is unclear. Taschen et al. (2020) found out that inoculation of *Q. ilex* seedlings with *T. melanosporum* did not enhance development in height or basal diameter after three years' growth in mesocosms conditions. On the other hand, Domínguez Núñez et al. (2006)observed a significant growth increase in *Q. ilex* seedlings inoculated with *T. melanosporum* spores as compared to non-inoculated ones after one year in the field. In our study, we detected a positive correlation of ectomycorrhizas and the extraradical mycelium of *T. melanosporum* with oak biomass, while no effect of AMF colonization on oak biomass was found. The chlorophyll content in *Q. ilex* leaves was unaffected by the presence of companion plants, either inoculated or non-inoculated. Our results agree with those of Taschen et al. (2020). The LMA values in oak leaves indicated that there was a higher competition for water in dual cultures as compared to oaks growing alone, but there were no significant differences among the treatments with and without inoculated AMF. Some studies in Mediterranean woody species and evergreen oak forests showed a

negative correlation between the LMA and water availability of their habitat, i.e., the higher the LMA, the lower the water content in soil (de la Riva et al., 2016; Salazar Zarzosa et al., 2021).

Quercus spp. are usually considered ectomycorrhizal, although AM fungal colonization seems to be also common, e.g., in section Lobatae (red oaks from North, Central, and South America) (Dickie et al., 2001). Dual mycorrhizal status is beneficial for the nutritional requirements of oaks in water-limited ecosystems (Querejeta et al., 2009) and as an insurance strategy in disturbed ecosystems when growing apart from other ectomycorrhizal trees (Teste et al., 2020). Smith et al. (1998) and Dickie et al. (2001) found out that AMF infection in Pinaceae and *Q. rubra*, respectively, is most successful when a predominately AM host is present. Previous studies have suggested that the effects of AMF on Quercus spp. may be related to plant survival rather than growth effects (Watson et al., 1990). We are not aware of other studies examining the ecology of AM fungal colonization and growth effects, specifically on the *Quercus* species of section Ilex. In our study, truffle-oaks growing alone did not show AM fungal colonization, whereas those growing together with arbuscular mycorrhizal MAPs showed an elevated root AM colonization (over 70%). This was coupled with a sharp reduction in truffle ectomycorrhizas, indicating both the strong competition of fungal mycorrhizal types and that dual *Q. ilex* mycorrhization is likely to occur in nature.

In our experimental conditions, MAPs also suffered from the competition of truffle-oaks, showing a significant decrease in growth and number of spikes (lavender), when comparing AM-MAPs growing alone with those growing together with truffle-oaks. González-Armada et al. (2010) reported numerous vascular plants growing inside the truffle brûlés, both in plantations and wild areas, including the MAPs *Lavandula latifolia* Medic. and *Thymus vulgaris* L., despite the truffle's allelopathy that inhibits the plant growth and coverage. However, most showed dwarfism and some were unable to complete their life cycle. Moreover, the diversity of ectomycorrhizal fungi and vascular plants were significantly reduced inside the brûlé.

Our results also show a protective effect of AMF in inoculated MAPs co-cultured with truffle-oaks, in which the former grew significantly more than the non-inoculated ones. This finding is consistent with numerous studies that have proven the beneficial effects of AMF on MAPs' tolerance to abiotic stresses, plant biomass, and essential oil content (Marulanda et al., 2007; Prasad et al., 2011; Camprubi et al., 2015; Amiri et al., 2017; Amanifar & Toghranegar, 2020; Israel et al., 2022). Some of the intercropping experiences in truffle orchards in Southern France included co-cultivated lavender rows and showed strong competition with truffle production (Geoffroy et al., 2018; Martin-Chave, 2019). According to our results, lavender growth is strongly affected in the presence of T. *melanosporum*, but this effect can be significantly reduced when inoculated with AMF.

In our study, similar to that observed for truffle ectomycorrhizas, the presence of MAPs in dual cultures significantly lowered *T. melanosporum* mycelium in the soil, making it almost undetectable when the companion MAPs were inoculated with native AMF, which lead us to reject our third hypothesis. The results from molecular analyses also showed a significant negative correlation between AM fungal biomass and the mycelium of *T. melanosporum* in the soil. Taschen et al. (2020) found out that the presence of some companion plants, such as *T. vulgaris* and *Festuca ovina* L., empirically associated by the orchard's owners with better truffle production, had no particularly positive effect on *T. melanosporum* mycelium abundance in rhizotrons. However, the companion plants were not inoculated and the abundance of *T. melanosporum* mycelium in the soil of inoculated truffle-oaks at the end of the experiment was almost three times that obtained in our study.

The mesocosm conditions allowed us to establish replicated experiments while maintaining some of the key dimensions of the natural systems to be studied (soil, companion plants, truffle colonization, and AMF colonization), which allowed us to infer causal relationships from observable effects (Crossland & La Point, 1992; Schindler, 1998). However, possible limitations derived from soil sterilization and the density restrains regarding plant growth (Dong et al., 2022) make it necessary to conduct further studies to confirm our obtained results under natural field conditions.

5. Conclusions

Co-culturing truffle-oaks and MAPs revealed strong interactions at both the plant and the fungal level under controlled conditions. Co-cultured truffle-oaks and MAPs, especially lavender, significantly reduced their growth as compared to their respective monocultures. However, this negative effect was buffered by their colonization with native AMF. In co-cultures, the AMF from MAPs likely competed with the *T. melanosporum* that was readily forming ectomycorrhizas with oaks, and in fact, a significant reduction in oak ectomycorrhizal percentage was observed. On the other hand, MAPs benefited from their associated arbuscular mycorrhizal fungi that were unaffected by co-culturing with oaks and promoted aromatic growth and, contrary to what was expected, also outcompeted the extraradical mycelium of *T. melanosporum* in soil. Overall, our results indicate that managing AMF in truffle plantations is an important factor to be considered when establishing the dual cultures of plant species and mycorrhizal types in intercropping systems.

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Abbreviations

AM	Arbuscular mycorrhizal
AMF	Arbuscular mycorrhizal fungi
ECM	Ectomycorrhizal
LMA	Leaf mass per area
MAPs	Medicinal and aromatic plants

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7. Supplementary material

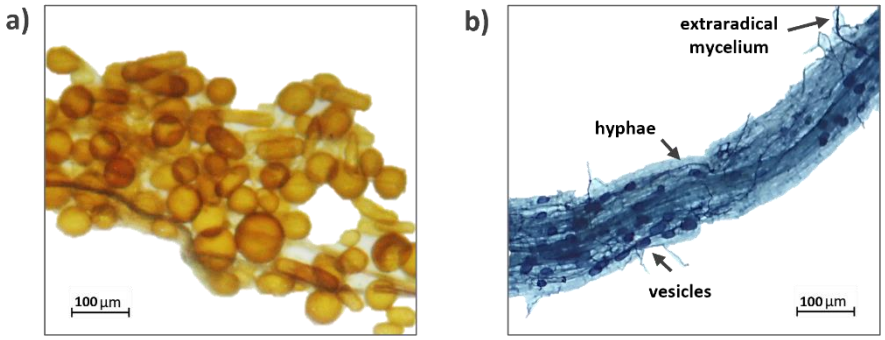


Figure S1 Stereomicroscope photographs of arbuscular mycorrhizal fungi (AMF) structures **(a)** Inoculum of *Glomus* spp-type spores isolated from native plants within the brûlés. (**b)** Root fragment of mycorrhizal lavender stained with trypan blue.

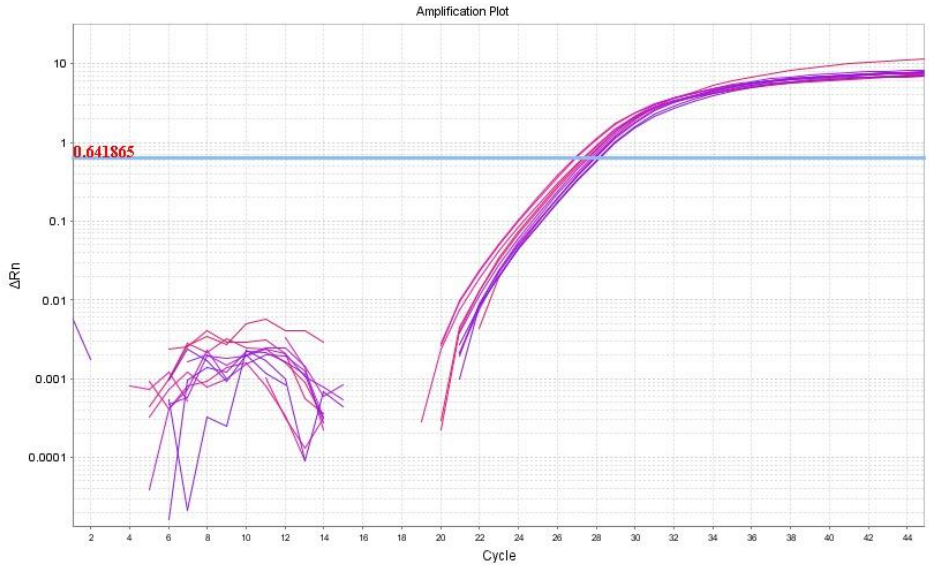


Figure S2 Amplification plot of fungal ITS1 multicopy gene. In the qPCR assay were used samples from different treatments and different initial DNA concentrations. Samples were fitted to equal concentrations to test whether the unknown number of ITS1 copies in a sample was related to its DNA concentration. Mean C_T values in the established threshold (0.641865) was 27.56 ± 0.42 standard deviation.

Table S1 Physical-chemical properties of the soil used in the compatibility assays. Organic matter was measured by Walkey-Black method. Elements were determined by spectrophotometry, after ammonium acetate (*) or diethylenetriaminepentaacetic acid (DTPA) (‡) soil extraction. Texture was determined by the Bouyoucos method.

Analysis	Result
Gravimetric moisture 105°C	<1.000 %
pH (ext. 1:2.5 v: v, H_2O)	8.5
Electrical Conductivity 25°C (ext. 1:5 v: v, H_2O)	0.173 dS m^{-1}
Organic Matter	1.41 %
$N\text{-}NO_3$	12.9 mg kg^{-1}
P (Olsen)	9.8 mg kg^{-1}
K*	149 mg kg^{-1}
Ca*	7304 mg kg^{-1}g
Mg*	167 mg kg^{-1}
Na*	27 mg kg^{-1}
Fe‡	4.505 mg kg^{-1}
Mn‡	5.787 mg kg^{-1}
Zn‡	3.881 mg kg^{-1}
Cu‡	0.322 mg kg^{-1}
Al‡	0.317 mg kg^{-1}
Pb‡	3.256 mg kg^{-1}
Cd‡	0.018 mg kg^{-1}
Ni‡	0.125 mg kg^{-1}
B‡	0.037 mg kg^{-1}
Sand	52 %
Clay	21.4 %
Lime	26.60 %
Stoniness	58.2 %

Table S2 Primer pairs tested in this work: specific primers to amplify arbuscular mycorrhizal fungi (AMF) genes and generic fungal primers tested as endogenous control (housekeeping genes) for relative quantification (qPCR $\Delta\Delta C_T$). The asterisks indicate the primers finally used in the experiment for the quantification of AMF relative to the total amount of fungi.

	Primer pairs	Target gene	Reference
AMF specific	AMG1F/AM1*	18S rRNA	(Bodenhausen et al., 2021)
Endogenous control	ITS1F/ITS2*	ITS1 region	(Toju et al., 2012)
	GAP_f1/GAP_r3	GAPDH	(Bodenhausen et al., 2021)
	GH63IF/GH63IR	GH63	(Pérez-Izquierdo et al., 2017)
	SPRYpF/SPRYpR	SPRYp	(Tao et al., 2016)
	Vps26F/Vps26R	Vps26	(Tao et al., 2016)
	RasF/RasR	Ras	(Tao et al., 2016)
	T1/Bt2b	β-tubulin	(Tao et al., 2016)
	Bt2a/Bt2b	β-tubulin	(Tao et al., 2016)

Table S3 Truffle-oak's growth variables. Treatment effect on truffle-oaks growing alone and co-cultured with medicinal aromatic plants either inoculated or non-inoculated with arbuscular mycorrhizal fungi (AMF). Variables were log (∞) transformed when necessary to meet the requirements to perform the ANOVA analysis (ANCOVA in the case of shoot dry weight), and significant values are highlighted in bold (P < 0.05). LMA=Leaf Mass per Area.

Truffle-oak variables	term	df	Sum sq	Mean sq	F-statistic	p-value
Shoots dry weight$^{\infty}$	treatment	6	1.715	0.286	18.704	**<0.0001**
	initial height	1	0.100	0.100	6.574	**0.0141**
	Residuals	41	0.626	0.015		
Roots dry weight$^{\infty}$	treatment	6	0.258	0.043	1.354	0.2554
	Residuals	42	1.336	0.032		
Shoot to root ratio$^{\infty}$	treatment	6	2.507	0.418	9.215	**<0.0001**
	Residuals	42	1.905	0.045		
Height$^{\infty}$	treatment	6	0.136	0.023	5.064	**0.0005**
	Residuals	42	0.188	0.004		
Diameter$^{\infty}$	treatment	6	0.102	0.017	3.442	**0.0074**
	Residuals	42	0.208	0.005		
LMA	treatment	6	6.858	1.143	3.962	**0.0031**
	Residuals	42	12.118	0.289		
Chlorophyll content	treatment	6	141.280	23.547	0.503	0.8023
	Residuals	42	1965.141	46.789		

Table S4 Medicinal and aromatic plants´ (MAPs) growth variables. Effect of arbuscular mycorrhizal fungi (AMF) on inoculated vs non-inoculated MAPs co-cultured with truffle-oaks, analysed by ANOVA; significant values are highlighted in bold (p<0.05).

Species	MAPs variables	term	df	Sum sq	Mean sq	F-statistic	*p*-value
Lavender	Shoots dry weight	AMF	1	231.227	231.227	32.116	**0.0001**
		Residuals	12	86.397	7.200		
	Roots dry weight	AMF	1	23.049	23.049	4.294	0.0605
		Residuals	12	64.418	5.368		
	Shoot to root ratio	AMF	1	0.805	0.805	1.682	0.2190
		Residuals	12	5.744	0.479		
	Height	AMF	1	58.018	58.018	2.244	0.1600
		Residuals	12	310.230	25.853		
	Lavender spikes	AMF	1	42.875	42.875	43.713	**0.0000**
		Residuals	12	11.770	0.981		
Sage	Shoots dry weight	AMF	1	104.261	104.261	11.801	**0.0049**
		Residuals	12	106.016	8.835		
	Roots dry weight	AMF	1	82.889	82.889	10.693	**0.0067**
		Residuals	12	93.021	7.752		
	Shoot to root ratio	AMF	1	0.131	0.131	1.378	0.2633
		Residuals	12	1.142	0.095		
	Height	AMF	1	103.143	103.143	9.691	**0.0090**
		Residuals	12	127.714	10.643		
Thyme	Shoots dry weight	AMF	1	24.314	24.314	5.004	**0.0450**
		Residuals	12	58.307	4.859		
	Roots dry weight	AMF	1	26.714	26.714	3.125	0.1025
		Residuals	12	102.598	8.550		
	Shoot to root ratio	AMF	1	0.090	0.090	0.201	0.6618
		Residuals	12	5.368	0.447		
	Height	AMF	1	1.341	1.341	2.036	0.1791
		Residuals	12	7.905	0.659		

Table S5 Quantification of arbuscular mycorrhizal (AM) extraradical mycelium in soils with truffle-oak growing together with AM lavender, sage or thyme. AM mycelium quantification is relative to each species´ control i.e., AM-lavender, AM-sage or AM-thyme growing alone, in which the quantity of AMF was considered as 100. Data are means ± SE of relative gene expression and were calculated by $\Delta\Delta C_T$ method.

Treatment	Relative quantification of AMF
truffle-oak + AM-lavender	36.2 ± 9.5
truffle-oak + AM-sage	36.4 ± 6.1
truffle-oak + AM-thyme	33.4 ± 9.6

Table S6 Percentage of arbuscular mycorrhizas (AM) in roots of medicinal and aromatic plants (MAPs) growing alone or with truffle-oaks. Data are means ± SE.

Treatment	AMF mycorrhizas (%)
AM-MAPs	57 ± 5.1
truffle-oak + AM-MAPs	65.6 ± 2.5

Chapter 5

Ecosystem services provided by truffle–producing systems in Spain: stakeholders perception and prioritisation

Manuscript under preparation for publication in:

Ecosystem Services

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1. Introduction

Edible fungi are greatly appreciated for their high nutritional value, the pleasant organoleptic properties, as well as their medicinal properties, and they have been collected in the wild since prehistoric times (Thu et al., 2020; Assemie & Abaya, 2022; Hamza et al., 2024). Among the edible fungi, a special interest has been drawn in truffles and particularly in black truffle, *Tuber melanosporum* Vittad., one of the most appreciated edible fungi in the market (Román & Boa, 2004; Reyna & Garcia-Barreda, 2014; Büntgen et al., 2017). Black truffle is an ectomycorrhizal fungus that lives in association with the roots of woody perennial plants (Smith & Read, 2008). It is naturally harvested in Europe, mainly in the Mediterranean forests of Spain, France, and Italy (Reyna & Garcia-Barreda, 2014), and it is considered as a culinary delicacy.

The rural exodus taking place in Mediterranean countries since mid-20th century motivated a decrease of traditional practices like clearing of forest understory or shepherding (Samils et al., 2008). As a result, forest cover increased and habitats were maintained by moderate levels of human interference, reducing in this way the open woodland conditions suitable for the truffles to thrive (Etienne et al., 1998; Debussche et al., 1999; Reyna-Domenech & García-Barreda, 2008). Forest-pasture transitional areas have gradually disappeared (Varela et al., 2020; Infante-Amate et al., 2022), and wild truffle production gradually declined (Samils et al., 2008). Under these circumstances, the success in inoculating host trees with *T. melanosporum* in the 1970s (Murat, 2015) did open the door to the species cultivation. Nowadays, black truffle cultivation sources the largest part of marketed truffles (Le Tacon et al., 2014).

Either in wild forests or in orchards, truffles grow belowground and they are harvested with the help of trained potting dogs to spot them and with specific tools to extract truffles from the soil (Oliach et al., 2021). Truffles are consumed either as a fresh product or processed as an ingredient for manufactured food products (Oliach et al., 2021) and, due to their unique and intense aroma, they are one of the most expensive foods in the world (Šiškovič et al., 2021). Black truffle cultivation is seen as an opportunity to revitalise the marginal rural areas where the fungus dwells, given the high agricultural income that it generates (Reyna-Domenech & García-Barreda, 2008; Therville et al., 2013; Bonet et al., 2014). Indeed, truffle yields may contribute substantially to rural economies of some southern Europe's countries (Büntgen et al., 2017), with documented paradigmatic cases of success in marginal depressed regions of inland Spain (Samils et al., 2008).

Beyond these pecuniary outcomes linked to the provisioning service dimension, truffle-growing ecosystems can contribute to the conservation of biodiversity and landscape heterogeneity (Therville

et al., 2013). The establishment of open woodland plantations for truffle production maintains landscape patchiness and, hence, provides suitable habitats for biodiversity conservation of species that are threatened by continuous forest cover in Mediterranean abandoned habitats (Reyna-Domenech & García-Barreda, 2008; Chevalier et al., 2010). Open landscapes are also able to provide fire regulation services, and truffle plantations with wide spacing among trees along with the "truffle brûlé" formation (a phenomenon where the fungus suppress the understory vegetation) are considered as potential firebreaks (Samils et al., 2008). In addition, truffle cultivation may represent a suitable option for post-fire restoration, enabling soil protection and soil structure enhancement (Martínez De Aragón et al., 2012).

Truffle ecosystems as agroforestry systems provide regulating services and they are considered to preserve and promote the soil quality (Guillot et al., 2021). Indeed, through trufficulture, limited size and marginal field plots can be cultivated, and the soil erosion risk can be, therefore, reduced (Reyna-Domenech & García-Barreda, 2008). Furthermore, mycorrhizal fungi like truffles form symbiotic relationship with their tree-hosts they play a crucial role in carbon sequestration, both in soil and trees (Treseder & Allen, 2000; Egli, 2011; Lindahl & Tunlid, 2015). As previously seen in **Chapter 2** and **Chapter 3**, black truffle and other fungi form different trophical groups contribute to soil functioning through their direct or indirect participation in carbon and nutrient cycling.

In terms of cultural services, truffle and truffle-related activities play a paramount role in the local cultural heritage of truffle regions (Therville et al., 2013) where cultivating and hunting truffles is an integral part of the local identity (Samils et al., 2008). Moreover, gastronomic routes dedicated to truffles and truffle festivals have promoted agritourism all over the world (Lefevre, 2012; Berch, 2020; Star et al., 2020; Calvo et al., 2022; Di Gregorio et al., 2022).

The heterogeneous landscapes, like those created by open truffle plantations, have been shown to attain higher social appreciation than more homogeneous landscapes (Franco et al., 2003; Dramstad et al., 2006; Tagliafierro et al., 2013). Although human perception of the landscape and its beauty varies depending on personal background and knowledge (Scarpa et al., 2000; Nielsen et al., 2007; Gundersen & Frivold, 2008), more heterogenous landscapes with open forest structures have been found to be generally preferred over homogenous closed canopy systems (Blasco et al., 2009).

Under these terms, the interaction between people and natural systems leading to the coproduction of ecosystem services (ES) (Fischer & Eastwood, 2016; Gissi & Garramone, 2018; Raymond et al., 2018) may be particularly relevant in truffle ecosystems, where human intervention is required for their conservation and enhancement. From this coproduction perspective,

disentangling the perception of ES by local residents is key to understand how much these services contribute to the societal wellbeing (Targetti et al., 2020).

In Spain, truffles are not so central to traditional cuisine and tradition as they are in France or Italy, although there is an extensive natural habitat for them. The interest in truffles developed later, when truffle cultivation and broader consumption were introduced in Spain as a result of the increasing market demand in other European countries (Samils et al., 2008).

The Delphi study conducted by Oliach et al. (2021) reported the most salient threats of the truffle sector evaluated by experts in the main producing Mediterranean countries, unveiling significant differences between them. Spanish experts considered relevant the potential impact of pests and diseases affecting truffle plantations, most likely linked to the increase in truffle plantation size and the large continuous areas with this crop, which has caused their appearance (Martín-Santafé et al., 2014). In this context, intercropping systems in truffle plantations may contribute to diversify the system, improving its resilience against pest and diseases while providing an extra source of economic income. Previous studies, together with the empirical knowledge of some truffle growers, have shown ecological evidence on the interactions of truffle-inoculated plants with specific companion plants (Taschen et al., 2020; Barou et al., 2023) and their insights can be useful for applying intercropping systems in truffle plantations. This practice, however, is associated with constraints such as increased machinery and labour costs (Low et al., 2023).

This study aimed to disentangle the importance that ecosystem services, ranging from provisioning, regulating and cultural dimensions, have for stakeholders directly involved in truffle culture in Spain, i.e. forest owners, researchers and technicians, and truffle processing operators. Furthermore, we aimed to understand the main barriers for truffle cultivation, as well as the key reasons that would encourage the implementation of intercropping systems in truffle plantations. For this purpose, we conducted an online survey addressed to the aforementioned stakeholders and employed the Analytical Hierarchic Process (AHP), which performs a pairwise comparative analysis (R. W. Saaty, 1987; T. L. Saaty, 2001).

Furthermore, we were interested in understanding whether the environmental attitudes of these stakeholders were related to the importance they attached to the different ES provided by truffle-producing systems and whether some patterns could be disentangled in this respect. For this purpose, we employed the widely use scale of the New Ecological Paradigm (NEP) (Dunlap et al., 2000) that measures general beliefs about the relationship of human beings with the environment, and we searched for joint patterns of ES prioritisation and environmental values through cluster analysis.

By mapping the perceptions on ES and environmental values supplied by truffle-producing systems, we aimed to signal key areas for policy and management intervention. To our knowledge, this is the first study addressing specifically the perceptions of stakeholders directly involved in truffle culture for the ES provided by these systems. Our results can contribute to inform the development of policies and strategies towards the enhancement and sustainability of these systems.

2. Materials and Methods

2.1. Case study area

The study targeted the main truffle-producing areas in Spain, which are mainly located at the central and north-east parts of the country (**Fig. 1**).

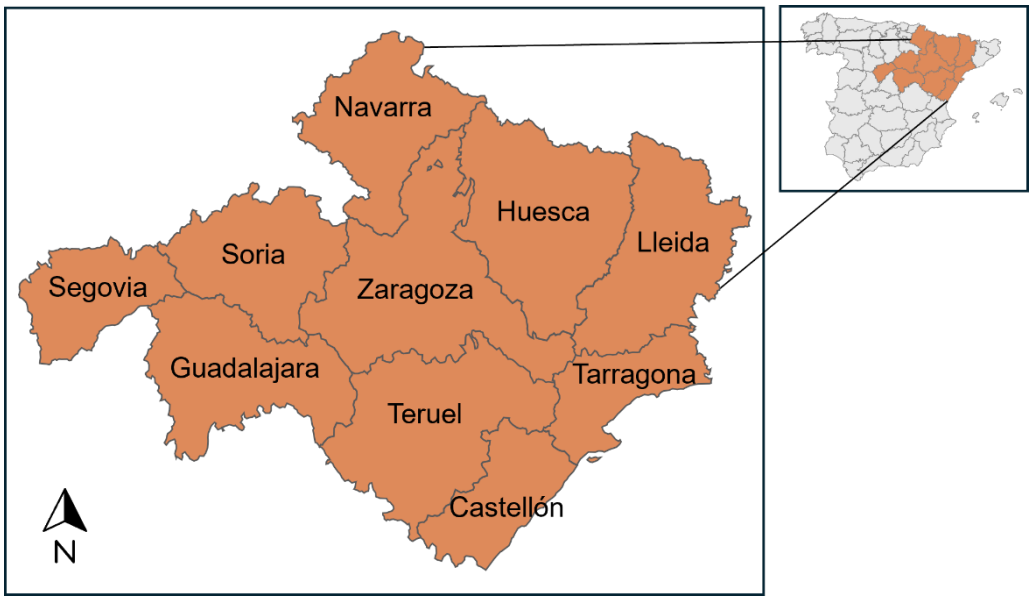


Figure 1 Provinces of Spain targeted by our study, where truffle-producing systems are mainly located. Map created in www.mapchart.net and edited by the authors.

2.2. Conceptual and methodological framework

A number of different typologies or ways of classifying ecosystem services are available, including those used in the Millennium Ecosystem Assessment (Millennium Ecosystem Assessment, 2005) and the Economics of Ecosystems and Biodiversity (TEEB, 2010). Several more recent examples include the Common International Classification of Ecosystem Services (CICES, Haines-Yong and Potschin, 2012; Haines-Young et al., 2016), developed by the European Environmental Agency and the system of Nature's Contributions to People (NCP; Pascual et al., 2017), and used by the Intergovernmental Panel on Biodiversity and Ecosystem Services (IPBES).

For this study, we adopted the latest version of CICES (v.5.1) (Haines-Yong & Potschin, 2018) in order to identify and characterise potential ecosystem services provided by the truffle-producing systems. CICES is a hierarchical framework that classifies the contribution of ES to human well-being into those related to the provisioning of material and energy needs, the regulation and maintenance of the environment for humans, and the non-material characteristics of ecosystems that affect physical and mental states of people, i.e. their cultural significance. We selected CICES because it offers a relatively high level of detail (the highest number of ES categories among the classifications already mentioned) in a nested hierarchical structure of 'taxonomical levels' (Czúcz et al., 2018).

Particularly important for our work, this version of CICES (Haines-Yong & Potschin, 2018) includes a new class described as "Fire protection" ES (FPES) within the group 'Regulation of baseline flows and extreme events', which recognises the ecological and anthropic dimensions that underlie the service, i.e., the role of ecological features and the contribution of their outcomes (e.g., the reduction of fire incidence, intensity or rate of spread) to human protection, by mitigating or preventing damages (costs) caused by fires to humans (e.g. human health and safety) and their livelihoods (e.g. the usage of natural resources by people) (Sil et al., 2019).

In this study, we were interested in understanding the relative importance that a range of different ES has for the stakeholders. For this purpose, we employed the Analytical Hierarchic Process (AHP), a classification procedure often used in multicriteria decision analysis (MCDA), which performs a pairwise comparative analysis (R. W. Saaty, 1987; T. L. Saaty, 2008). AHP has found wide application in environmental decision-making (Schmoldt et al., 2001), especially when the stakeholders are involved in the process (Marques-Perez et al., 2014; Segura et al., 2014). This method encourages differentiation between criteria to a greater extent than simple rating methods and highlights inconsistencies in responses. Previous examples of its usage include sustainable forest management (Maroto Álvarez et al., 2013; Uhde et al., 2015), prioritisation of framework for managing invasive alien plants (Potgieter et al., 2018) for zoning areas of environmental fragility (França et al., 2019), or evaluation of ES delivered by urban parks (Jang-Hwan et al., 2020). More recent examples of Figueiroa et al. (2020) and Przewoźna et al. (2022) have employed AHP approach for the assessment of ES provided by protected areas and tree management in urban and rural areas, respectively.

AHP performs pairwise comparative analysis to derive weights through pairwise comparison processes on a 9-point scale, from the left with 9 (much greater importance of the left element of the pair), to the centre with 1 (equal importance of both elements of the pair) and to the right with 9 (much greater importance of the right element of the pair) (see an example in **Fig. S1**). By judging two elements at the same time, this relative weighting method may foster the precision of the evaluation, and thus, the prioritisation of these elements (T. L. Saaty, 2013; Ishizaka & Siraj, 2018), potentially

reducing the level of non-differentiated weights as reported for rating methods (Ebner et al., 2022; Maestre-Andrés et al., 2016; Schmidt et al., 2017).

In order to disentangle to what extent these individual prioritisations of ES were related with their environmental attitudes, we employed the New Environmental Paradigm (NEP) proposed by (Dunlap et al., 2000). NEP is a validated scale, seeking to measure the environmental worldview among groups of people. The environmental worldview represents a common perception regarding the human interaction with the environment that influences the use of natural resources and the behaviour with nature (Naidoo & Gasparatos, 2023). Typically, anthropocentric and ecocentric worldviews can be disentangled within the five hypothesised facets of an ecological worldview (Dunlap et al., 2000): limits to growth, anti-anthropocentrism, the fragility of nature's balance, rejection of human exceptionalism, and the possibility of an ecocrisis. To measure this worldview, 15 statements are provided about the relationship between humans and the environment (see **Table S1**), and the respondents are asked for their degree of agreement with each one of these statements, using a 6-point Likert scale (from 1 – strong disagreement to 6 – strong agreement). To reduce response bias, odd-numbered items are phrased in such a way that respondents' agreement would indicate a pro-environmental worldview, while the disagreement with the even-numbered items would indicate the same worldview. Following the recommendation done by Dunlap et al. (2000), we factor-analysed the entire set of items to determine the dimensions emerging in our particular study case.

2.3. Questionnaire design

A semi-structured questionnaire was designed to gain understanding of the most salient ES provided by truffle-producing systems (see **Appendix**). The semi-structured questionnaire was given to three local experts through face-to-face interviews. They all shared a technical and academic profile and deep knowledge of truffle-producing systems in Spain. In this questionnaire we presented to experts an initial list of ES provided by truffle-producing systems, including 8 provisioning, 10 regulating and 6 cultural services, and we asked them to give their feedback on the relevance of these ES according to their preferences. We also asked them to consider how different stakeholders (i.e., truffle farmers, wild truffle collectors, truffle specialised researchers or technicians working in the local administration, owners of truffle nursery and employees who prepare/sale products made with truffle in restaurants or companies) would ponder the different ES.

In addition, we presented to the experts the three types of truffle-producing systems, i.e. truffle productive forests, truffle plantations and intercropping truffle plantations, and asked them to provide a reasoned list of ecologic, social and economic pros and cons for each truffle-producing system considering their own perspective, as well as that of other stakeholders. Finally, we provided a list of

farming practices and environmental and administrative factors that would facilitate the adoption of a multifunctional truffle-producing system, and asked experts to evaluate them using a 5-step scale (from 1 – not at all important, to 5 – very important).

Based on the expert's inputs, we designed a close-ended questionnaire that was administered through an online survey and addressed to stakeholders in the truffle sector (see next section). The questionnaire started with a brief presentation of the study and its objectives, followed by questions about sociodemographic characteristics of the surveyed individuals (i.e., age, gender, stakeholder group).

The next part of the questionnaire included a brief definition of the term "ecosystem service" and the presentation of the seven ecosystem services related to the truffle-producing systems that were finally selected: 1) truffle production, 2) soil quality maintenance, 3) CO_2 sequestration enhancement, 4) fire risk reduction, 5) biodiversity maintenance, 6) landscape enjoyment, and 7) cultural heritage (**Fig. 2**, **Table 1).** Respondents were first asked to rate the importance of each ecosystem service using a 10-point Likert scale (1 – not all important, 10 – absolutely important), what also allowed them to gain familiarity with these ES.

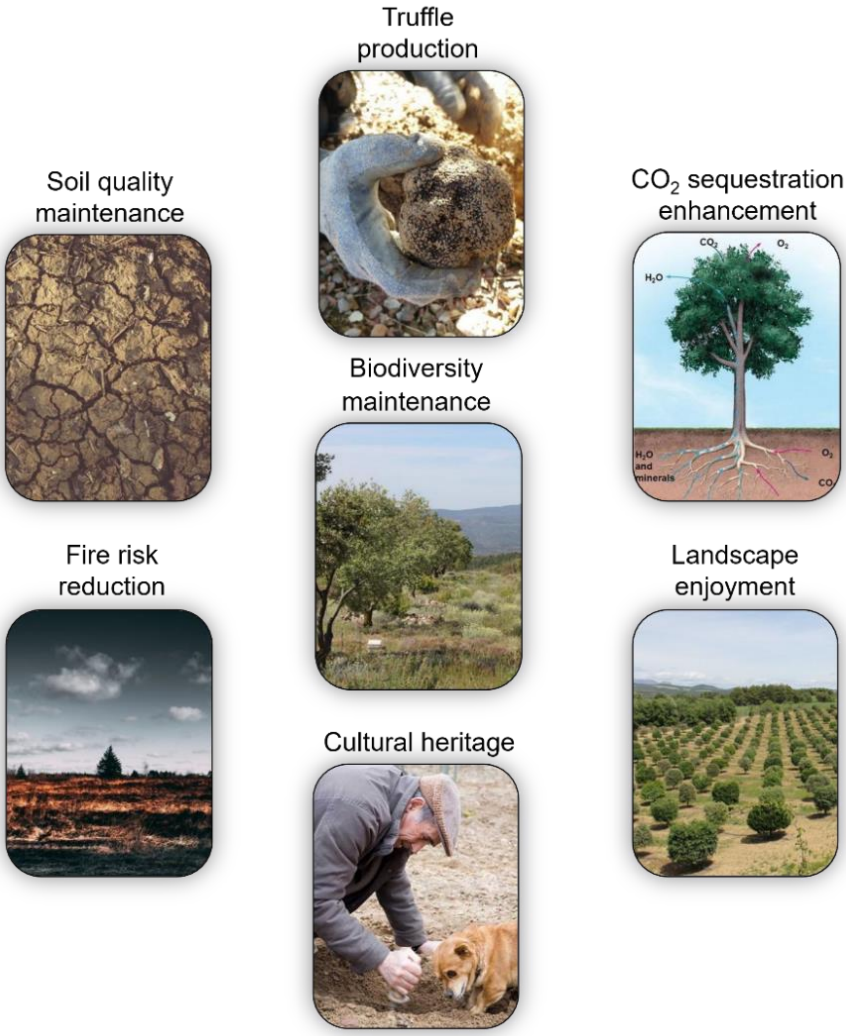


Figure 2 The ecosystem services related with the truffle production systems, as presented to the stakeholders.

Then, in order to unveil relative importance that the ES provided by truffle-producing systems had for the stakeholders in, we employed the AHP method, where the ES were displayed in pairs and the respondents were asked to exhaustively evaluate the importance of each ES in the paired comparison. They evaluated a total of 21 paired comparisons.

Table 1 The seven ecosystem services (ES) of truffle-producing systems that were evaluated by stakeholders in the questionnaire. The ES were selected according to those listed in CICES v5.1 and corresponded to the denoted code of CICES.

Ecosystem Service	Type of ES	Code	Description provided in the questionnaire
1. Truffle production (*Truffle*)	Provisioning	1.1.1.1	It is the quintessential service that truffle cultivation provides and can be measured in kg of truffles ha^{-1} $year^{-1}$.
2. Soil quality maintenance (*Soil*)	Regulation and maintenance	2.2.4.1	The radicular structure of the oaks and the presence of the truffle fungus and other microorganisms contribute to maintain soil fertility and structure.
3. CO_2 sequestration by soil and trees (*CO_2*)	Regulation and maintenance	2.2.6.1	Carbon is fixed in the soil of truffle plantations (i.e., microbial biomass, including that of truffle mycelium), additionally the carbon fixed by the trees.
4. Fire risk reduction (*Fire*)	Regulation and maintenance	2.2.1.5	The truffle-oak plantations may contribute to create landscapes with low fire risk because their structure hinders the progress of the fire in comparison with dense forests.
5. Provision of habitat for biodiversity maintenance (*Biodiversity*)	Regulation and maintenance	2.2.3.1	The truffle-producing systems provide the necessary conditions for the development of diverse species populations, such as birds, small mammals, and insects, contributing, this way, to maintain the biodiversity.
6. Aesthetic enjoyment of truffle plantation landscape (*Landscape*)	Cultural	3.1.2.4	The open landscape created by truffle plantations may be visually attractive, both for the residents and the visitors.
7. Cultural heritage linked with truffle hunting and truffle-related practices (*Cultural*)	Cultural	3.1.2.3	The cultivation practices and the truffle harvesting with the dog aid, as well as the tools used in this process and their names, are part of the cultural heritage of the truffle regions, and represent a cultural service linked with truffle-producing systems.

In addition, the stakeholders were asked to select the three most important difficulties/barriers from 11 topics related to truffle culture, by ranking them from the first to the third most important barrier (**Table 2**).

Table 2 The barriers/difficulties related to truffle culture that were under evaluation by stakeholders in the questionnaire.

Barrier/difficulty	Statement
1. Irrigation	Insufficient accessibility to irrigation at a reasonable cost.
2. Pests	Pests and diseases of truffle and/or truffle plants.
3. Tradition	Less culinary tradition compared to other countries (e.g., France, Italy).
4. Association	Individualised sector with very low associative culture.
5. Legislation	Existent legislation focused on forest management rather than on the agricultural aspect of truffle cultivation.
6. Land price	Increase of land price hindering new truffle plantations.
7. Establishment	Lack of economic incentives for the establishment of the truffle plantation.
8. Manpower	Lack of manpower due to rural abandonment.
9. Wild boar	Increase of wild boar populations that may damage the truffle plantations.
10. Enterprises	Lack of enterprises to incentivise local population into truffle cultivation.
11. Professionalism	Low professional competency of truffle producers.

Next, the questionnaire introduced multiple crop cultivation in truffle plantations where stakeholders were asked to select the three most important reasons that, in their opinion, would facilitate the adoption of an intercropping system instead of a truffle monoculture (**Table 3**).

Table 3 The incentives for truffle growers to implement a secondary crop that were under evaluation by stakeholders in the questionnaire.

Incentive	Statement
1. Profitability of secondary crop	The secondary crop is profitable.
2. Experience on secondary crop	The truffle grower has experience in the cultivation of the secondary crop.
3. Truffle cultivation improvement	A secondary crop may improve soil quality and attract natural enemies for biological control of pests of truffles and oaks.
4. Manpower availability	It is feasible to find the extra manpower needed for the field.
5. Extra income	With this practice, there is an opportunity to obtain an extra income from the secondary crop until truffle production initiates.
6. Tax allowances	There are tax allowances for truffle growers that implement intercropping.
7. Machinery availability	There is availability of adequate machinery to process the harvested crops.
8. Climate/soil adequacy	The climate and soil are adequate for a secondary crop production.
9. Field size for mechanisation	The size of the truffle plantation allows the mechanising operation of the secondary crop.

The last question in the survey collected the degree of agreement of the respondents according to a 6-point Likert scale with the 15 statements of the New Environmental Paradigm (NEP).

2.4. Data collection

We distinguished five different categories of stakeholders present in the truffle sector: truffle farmers, wild truffle collectors, truffle specialised researchers or technicians working in the local administration, owners of truffle nurseries and employees in truffle-processing.

The survey was administered online through the QuestionPro platform (QuestionPro Inc., 9450 SW Gemini Dr #62790, Beaverton; www.questionpro.com) during January and February 2023. Previously, we undertook 3 pilot surveys to test the understandability and ease of the online survey tool.

We followed a snowball sampling approach to distribute the survey among the desired sample of stakeholders linked to truffle-producing systems. We contacted researchers across the main locations of truffle-production in Spain, who were willing to contributing to our project, and they shared the survey link among their peers, members of truffle associations, academics and local people involved in the truffle sector.

A total of 217 stakeholders related to the truffle sector answered the survey. The responses where the questions about ES prioritisation through AHP were not filled in, were discarded for ulterior analysis. The final sample size was 135 responses. The demographic characteristics of the stakeholders are provided in the **Table S2**.

2.5. Data analysis

The mean ± SD of the ES rating was calculated. For the AHP analysis, the *ahpsurvey* R package (Cho, 2019) was used, and the relative importance of each ES was estimated by calculating the aggregated importance weights (i.e., the aggregated individual priorities of all stakeholders) with the geometric means method. The consistency ratio (CR) (R. W. Saaty, 1987) was calculated for each comparison matrix, determining how much the obtained results differ from purely random values, which indicates their reliability. This allowed us to identify inconsistent responses among the participants, i.e., contradicting ES priorities for individual judgments. Although the widely accepted threshold for consistency is CR ≤ 0.1, Ishizaka and Siraj (2018) suggested that higher thresholds are also valid, thus we considered responses with CR ≤ 0.2 as consistent (**Fig. S2**). A principal component analysis (PCA) was conducted on the individual prioritisation of ES as obtained from the AHP analysis to determine the main ES dimensions present in the data. The internal consistency (Cronbach's alpha),

sampling adequacy (Kaiser-Meyer-Olkin test; KMO) and sphericity (Bartlett´s test) were tested prior to PCA.

Similarly for NEP items, the data were first tested for Cronbach's alpha, KMO and Bartlett´s test. Then, to explore the dimensionality of the NEP Scale, a PCA with varimax rotation was conducted. The dimensions obtained from the two PCA (three on ES and five on NEP statements) were employed to conduct a hierarchical cluster analysis with the objective to unveil patterns in our sample of ES prioritisation and environmental awareness. The dimensions were scaled, the Euclidean distances between the coordinates were calculated and the hierarchical cluster was built with the "hclust" function of the *stats* R package, using the Ward linkage method. To determine the optimum number of clusters, the *NbClust* R package was used (Charrad et al., 2014).

Finally, we calculated the percentage of responses for each of the factors that would stimulate intercropping implementation on a truffle plantation and the ranking of the barriers related to truffle culture. All analyses were performed with the R software v.4.2.1 (R Core Team, 2022). The methodological overview of the study is resumed in **Fig. 3**.

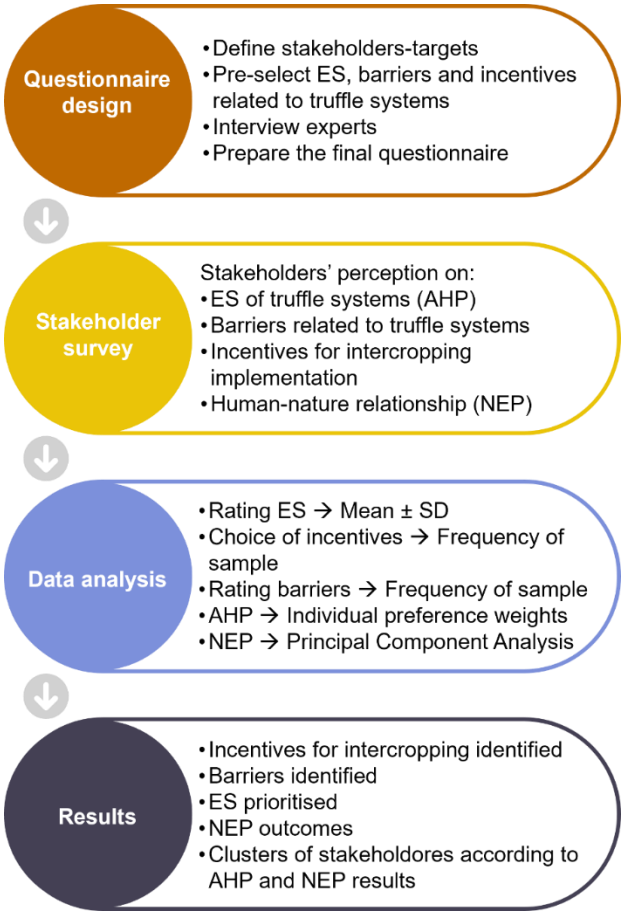


Figure 3 Methodological overview of the study aiming to assess the ecosystem services (ES), barriers and incentives for sustainable truffle-producing systems. AHP = Analytic Hierarchy Process, NEP = New Environmental Paradigm, SD = standard deviation.

3. Results

3.1. Rating and prioritisation of ES related to truffle-producing systems

The direct rating of ES in a scale from 1-10 showed that wildfire risk reduction, truffle production and soil quality maintenance obtained the highest estimates with average rating ± SD of 8.5 ± 2, 8.2 ± 2.2 and 8.1 ± 2.1, respectively (**Fig. 4**). Lower scores, but similarly high, were obtained for cultural heritage (7.9 ± 2.2), provision of habitats for biodiversity maintenance (7.9 ± 2.1) and CO_2 sequestration (7.8 ± 2.2) associated with truffle–producing systems. Lastly, the aesthetic enjoyment of the truffle plantation landscape was evaluated as the least important ES, with an average score of 6.7 ± 2.5.

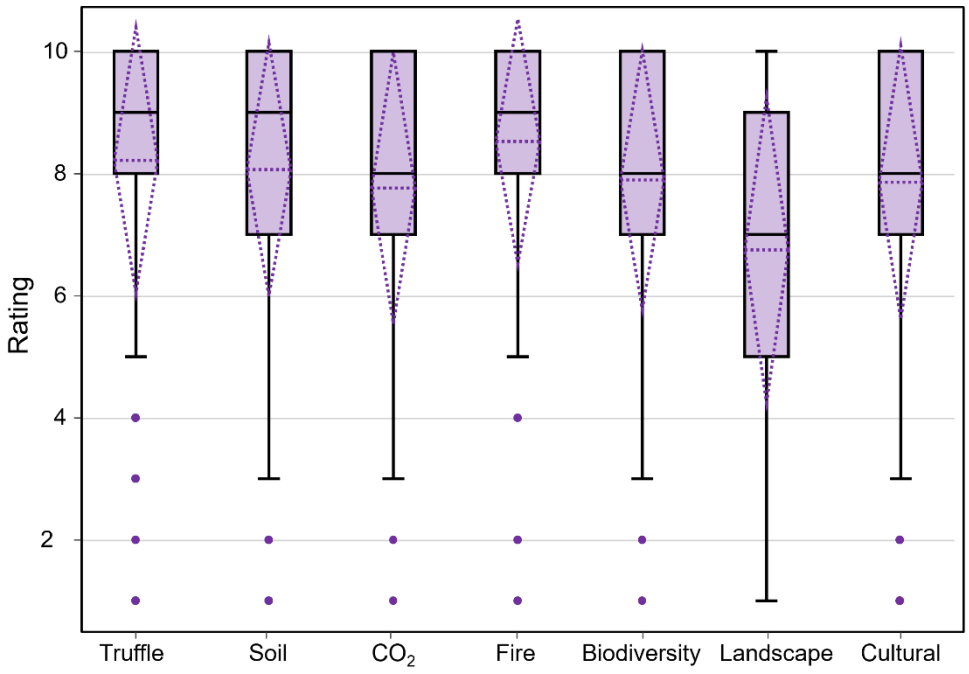


Figure 4 Rating (1–10) of seven ecosystem services provided by truffle ecosystems. Boxplots with min, max, median (horizonal solid line), quartiles, dashed horizontal line representing the mean and dashed rhombus representing the standard deviation (*N* = 135).

As regards the AHP results, out of the 135 participants, 130 passed the criterion of CR ≤ 0.2. Mean estimates of the prioritisation showed that truffle production was the ecosystem service attaining a higher importance, followed by wildfire prevention and the provision of habitats for biodiversity maintenance (**Fig. 5**). Soil quality preservation and CO_2 obtained similar estimates, only slightly higher that cultural heritage and, finally, the aesthetic enjoyment of the truffle plantation landscape scored the lowest.

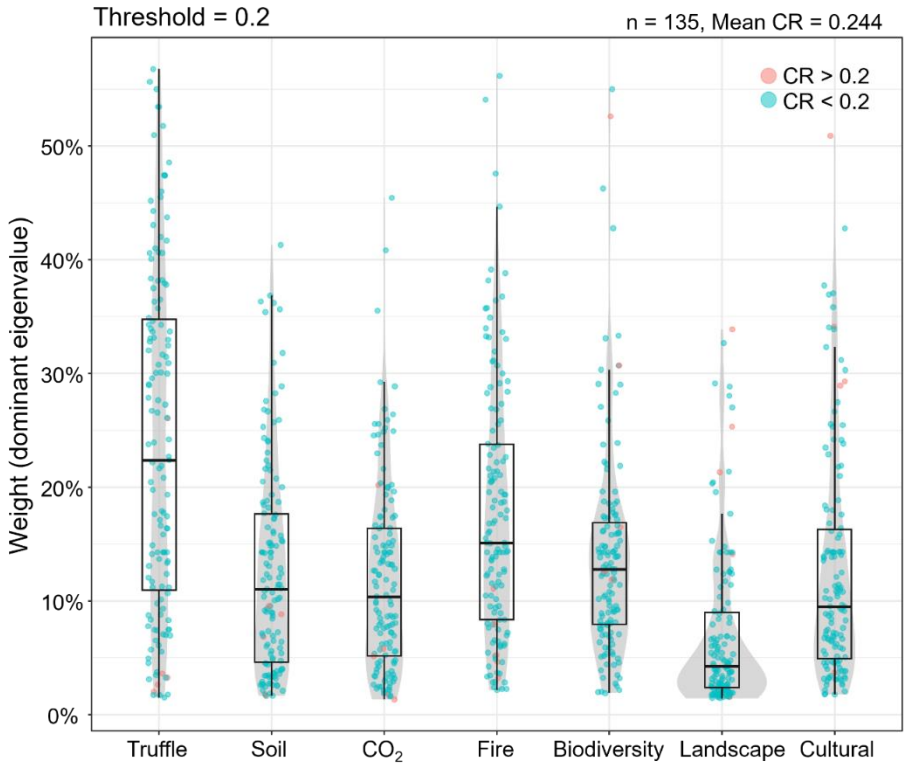


Figure 5 Overall prioritisation (preference weights) of seven ecosystem services provided by truffle-producing systems evaluated through Analytical Hierarchic Process (8 iterations, threshold = 0.2). Boxplots with min, max, median (horizonal solid line), quartiles and grey violins representing the distribution of the data.

3.2. Evaluation of the barriers related to truffle–producing systems

Among the total sample, 46 participants evaluated the importance of the difficulties that truffle growers usually encounter. The insufficient accessibility to irrigation at a reasonable cost was considered by far the most important barrier, chosen by around 60 % of the sample and ranked as the first most important barrier by half of them (**Fig. 6**). Pests of truffle plantations and less culinary tradition on truffles compared with other countries were ranked as important barriers by almost one third of the sample, followed by the low associative culture and the forest legislation applied to truffle cultivation. Chosen by less than 25 % of the sample, the high land price, the lack of economic incentives for the establishment of truffle plantations and the lack of manpower were also considered important barriers. Finally, the increase of wild boar populations, the lack of large enterprises to incentivise local population into truffle cultivation and low professional competency of truffle producers were ranked as the less important barriers, chosen by 15–18 % of the sample (**Fig. 6**).

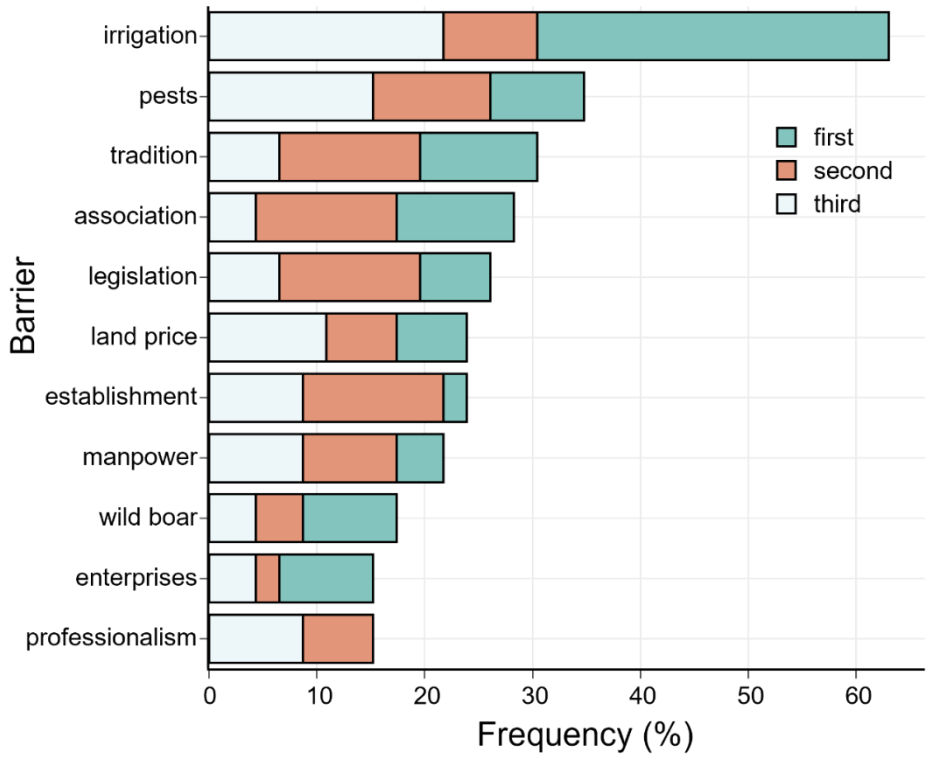


Figure 6 Percentage of sample (*N* = 46) ranking the main barriers related to truffle culture, from the first to the third most important barrier. See **Table 2** for the detailed explanation of the barriers.

3.3. Evaluation of incentives for intercropping implementation

One third of the total sample (*N* = 44) answered the question about the reasons that would motivate a truffle plantation owner to diversify their truffle plantation by introducing a secondary crop (e.g., aromatic plants, cereals, vineyard etc.). Obtaining extra income from a secondary crop and the improvement of the truffle cultivation conditions were the most frequent reasons chosen by the stakeholders, followed by the adequacy of environmental conditions, the profitability of the secondary crop and the sufficient field size for mechanisation (**Fig. 7**). Having previous experience on the cultivation of the secondary crop, availability of machinery that will process the harvested crop, manpower availability and tax allowances were chosen by less than 10 % of the sample as important reasons that would motivate the implementation of intercropping systems.

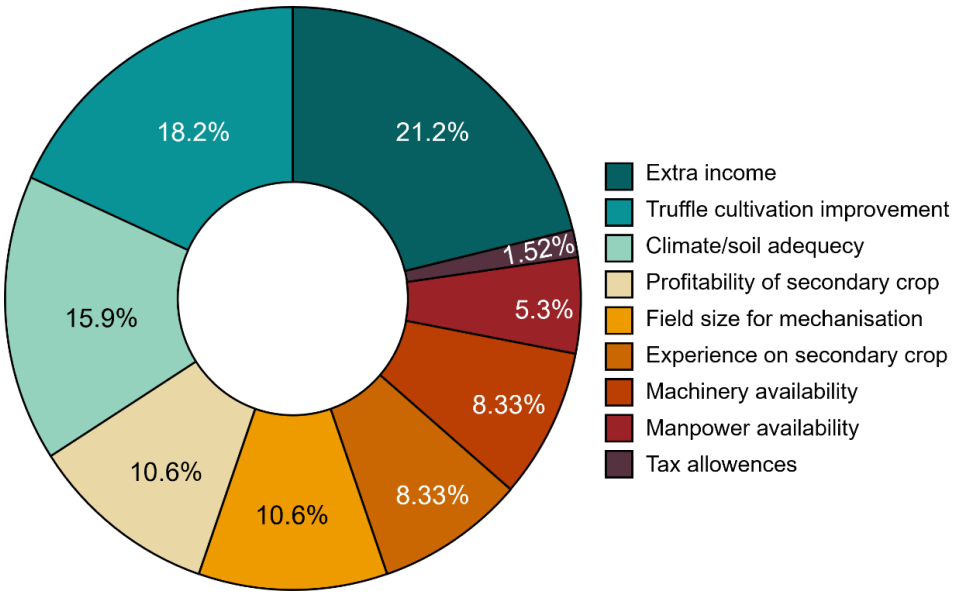


Figure 7 Percentage of sample (*N* = 44) choosing the factors that would stimulate intercropping implementation on a truffle plantation. See **Table 3** for the detailed explanation of the incentives.

3.4. Evaluation of New Environmental Paradigm

Regarding the exploratory analysis of the NEP Scale evaluated by 112 respondents, a PCA of the 15 statements was conducted. Since NEP 10 and NEP 11 did not meet the threshold criteria of minimal factor loadings and NEP 3 did not meet the commonality criteria (see details in Supplementary material, **Text S1**, **Table S3**, **Table S4**), these items were excluded from the dataset.

The remaining 12–item NEP was further assessed, and the Bartlett's test of sphericity (Chi–square$_{66}$ = 284.24, $p < 0.001$) and Kaiser–Meyer–Olkin test (KMO = 0.71) suggested that the data were appropriate for factor analysis. The Cronbach's alpha ($\alpha = 0.71$) showed that the 12–item NEP had a satisfactory level of internal consistency and reliability. Then, a new PCA was conducted and the loadings of the first five PCs with eigenvalues higher than 1 (Luo & Deng, 2008) and accounting for more than 68 % of the explained variance, were retained as the new factors (Hair, 2014) for varimax rotation (**Table S5**). The rotated loadings of each NEP variable showed values higher than |0.50| for at least one factor (**Table 4**) and communality values were also higher than |0.50| (**Table S6**).

The first two PCs or factors of the 12–item NEP explained almost 42 % of the total variance and showed a grouping of even and odd statements separately (**Fig. S3**). Typically, the agreement with the odd–numbered statements and the disagreement with the even–numbered ones indicate a pro–ecological worldview. Our analyses identified five PCs or factors where factors 1 and 3 show ecocentric or pro–environmental worldview whereas factors 2,4 and 5 show different aspects of anthropocentric worldview.

Table 4 Varimax rotation loadings of the 12–item New Environmental Paradigm (NEP) and groups of ecological overviews according to Dunlap et al. (2000).

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
Factor 1: Ecocrisis and rejection of limits to growth and exemptionalism					
NEP_5: Humans are seriously abusing the environment.	**0.826**	-0.123	0.126	-0.146	-0.12
NEP_9: Despite our special abilities, humans are still subject to the laws of nature.	**0.731**	0.137	0.099	0.033	-0.164
NEP_7: Plants and animals have as much right as humans to exist.	**0.706**	-0.035	0.292	-0.041	0.17
Factor 2: Rejection of limits to growth and exemptionalism and humans over nature					
NEP_6: The Earth has plenty of natural resources if we just learn how to develop them.	0.246	**0.768**	-0.205	-0.175	0.059
NEP_14: Humans will eventually learn enough about how nature works to be able to control it.	0.012	**0.661**	0.366	0.152	0.318
NEP_12: Humans were meant to rule over the rest of nature.	-0.361	**0.606**	-0.149	0.006	0.053
Factor 3: Vulnerability of nature´s balance and ecocrisis					
NEP_13: The balance of nature is very delicate and easily upset.	0.236	-0.064	**0.728**	-0.033	-0.073
NEP_15: If things continue on their present course, we will soon experience a major ecological catastrophe.	0.235	0.188	**0.704**	0.025	-0.398
NEP_8: The balance of nature is strong enough to cope with the impacts of modern industrial nations.	-0.105	0.402	**-0.68**	0.249	-0.055
Factor 4: Humans over nature					
NEP_2: Humans have the right to modify the natural environment to suit their needs.	-0.089	-0.041	-0.12	**0.904**	0.101
Factor 5: Exemptionalism and limits to growth					
NEP_4: Human ingenuity will ensure that we do not make the Earth unliveable.	0.059	0.172	-0.065	0.249	**0.786**
NEP_1: We are approaching the limit of the number of people the Earth can support.	0.413	-0.137	0.228	0.254	**-0.623**
Eigenvalue	2.209	1.679	1.868	1.085	1.36
% of variance	18.4	14	15.6	9	11.3
Cumulative %)	18.4	48	34	68.4	59.3

3.5. Clustering

The three varimax rotated dimensions of ES prioritisation and the five rotated dimensions of NEP items were jointly employed in a hierarchical cluster analysis resulting in three clusters (**Fig. 8**). Cluster 1 was composed by 52 individuals from the sample. This cluster allocated the highest importance to fire protection and biodiversity conservation (i.e., regulation and maintenance ES) amongst the three clusters (**Fig. 9a**). Individuals in this group scored also very high on the Factor 2 of the NEP analysis (**Fig. 9b**) that contained statements underlining the human domination over nature (**Table 4**). Therefore, we tagged this cluster as the fire–concern and human domination group (**Table 5**).

Cluster 2 gathered 41 individuals from the sample. Among the three clusters, individuals in this group attached the highest importance to the truffle production (provisioning ES), while scoring lowest on biodiversity maintenance, landscape enjoyment and cultural heritage (**Fig. 9a**). They showed positive scorings in Factors 1 and 3, dimensions resulting from the NEP that underlined an ecocentric worldview. This group also showed the most negative value for Factor 5 (**Fig. 9b**), which gathered "humans over nature" worldviews. Hence, we tagged this cluster as the truffle production–oriented and ecocentric group (**Table 5**).

Cluster 3 is the smallest one with 19 individuals belonging to this group. In this cluster, cultural heritage scored higher than truffle provision, while CO_2 sequestration and fire protection obtained the lowest importance amongst the three groups (**Fig. 9a**). Interestingly, this group showed negative scoring in Factor 1 (**Fig. 9b**) that brings together "humans over nature" statement with a dominating worldview. However, this group regarded positively dimension Factor 5 (**Fig. 9b**), where "humans exemptionalism" was expressed as using science and invention to overcome sustainability challenges. This group seemed to reject equally domination, as well as ecocrisis worldviews and seemed to be closer to a respectful coexistence with nature, where science and innovation may allow for socio–ecological coexistence. We tagged this group as the cultural–landscape aware group (**Table 5**).

Finally, although we thoroughly tested cluster membership considering variables such as the age, gender and whether the respondents were truffle plantation owners, to further profile the clusters, none of these variables resulted statistically significant to define cluster membership.

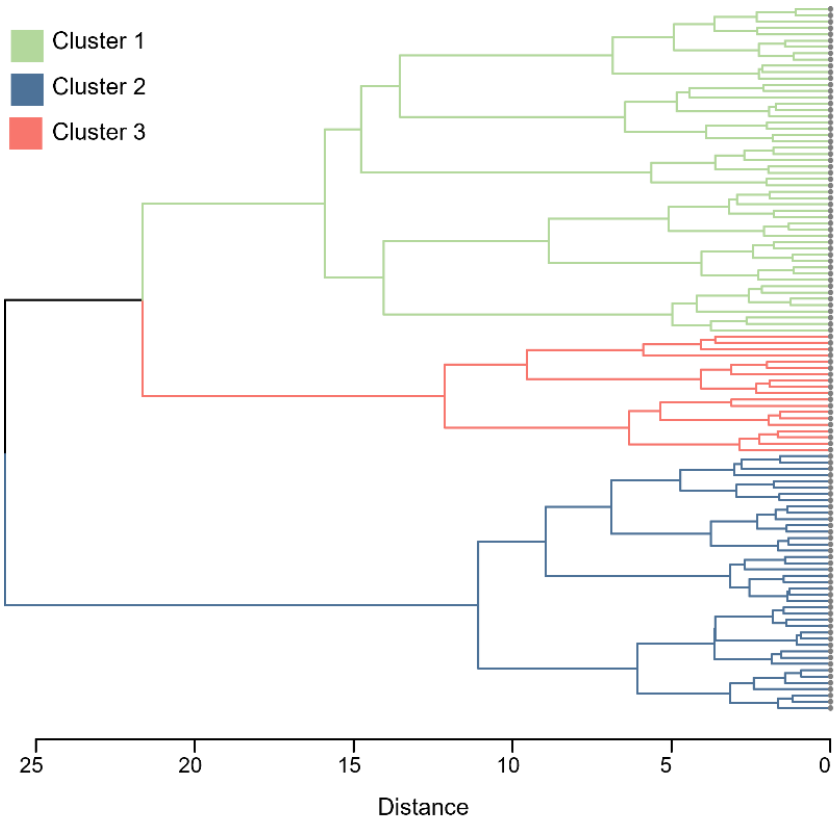


Figure 8 Dendrogram of hierarchical cluster analysis based on the rotated loadings of ecosystem services (ES) prioritisation (three varimax rotated dimensions), derived from the Analytical Hierarchic Process (AHP) analysis, and of New Environmental Paradigm (NEP) items (five varimax rotated dimensions). Cluster 1 (*N* = 52), Cluster 2 (*N* = 41), Cluster 3 (*N* = 19). The dissimilarity among clusters is represented by the value of Euclidean distance.

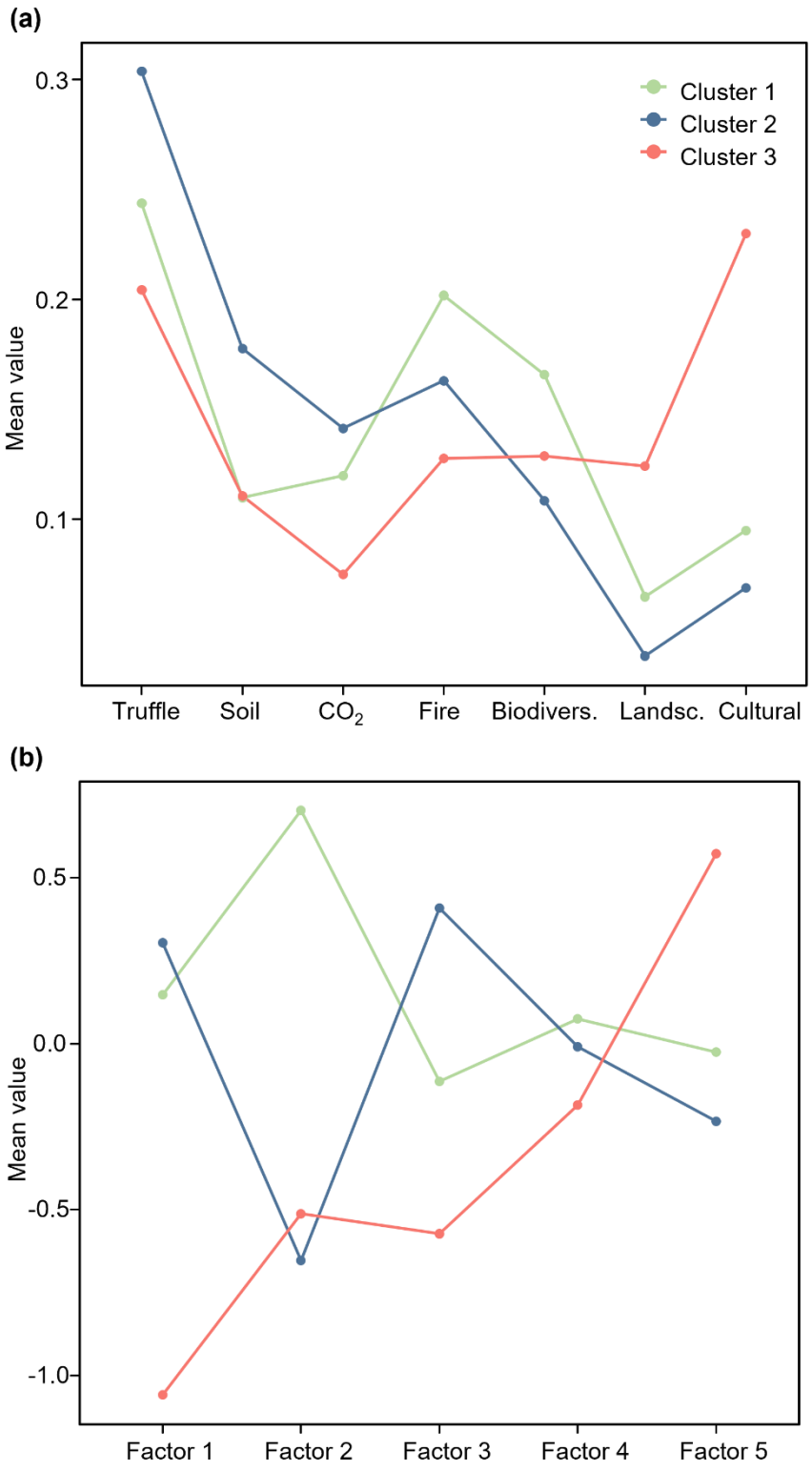


Figure 9 Parallel coordinates charts representing (**a**) mean ecosystem services (ES) prioritisation values derived from the Analytical Hierarchic Process (AHP) analysis and (**b**) mean factor values derived from varimax rotated components of the 12-item New Environmental Paradigm (NEP) across the different clustered groups. Biodivers. = Biodiversity, Landsc. = Landscape, Cluster 1 (*N* = 52), Cluster 2 (*N* = 41), Cluster 3 (*N* = 19).

Table 5 Mean values of ecosystem services (ES) prioritisation derived from the Analytical Hierarchic Process (AHP) analysis and of factors derived from varimax rotated components of the 12–item New Environmental Paradigm (NEP) for each cluster.

	Cluster		
	1. Fire–concern and human domination group	**2. Truffle production–orientated and ecocentric group**	**3. Cultural–landscape aware group**
Truffle	0.244	0.304	0.204
Soil	0.11	0.178	0.111
CO_2	0.12	0.141	0.075
Fire	0.202	0.163	0.128
Biodiversity	0.166	0.108	0.129
Landscape	0.065	0.038	0.124
Cultural	0.095	0.069	0.23
Factor 1	0.147	0.304	-1.059
Factor 2	0.702	-0.653	-0.512
Factor 3	-0.113	0.409	-0.573
Factor 4	0.075	-0.009	-0.185
Factor 5	-0.025	-0.234	0.573

4. Discussion

In this study, we assessed the perceived importance of seven ES, representing provisioning, regulating, and cultural services, associated with truffle producing systems and the biodiversity conservation of these ecosystems. The targeted stakeholders, who belonged to different landscape user groups but all related to truffle producing systems, shed light on the multifunctional aspect of these ecosystems. The multicriteria analysis method of AHP was used to prioritise the ES in a consistent way, while the NEP allowed to disentangle 5 dimensions related to ecological worldviews of these stakeholders. Finally, the cluster analysis joined patterns of ES prioritisation and ecological worldviews.

4.1. Provisioning aspect of truffle–producing systems

As it would be expected, the provisioning service of truffle production was ranked as the first most important ES by the stakeholders. The truffle production sector has experienced a remarkable growth in the last years as a consequence of the successful truffle species cultivation and the increasing truffle demand in the market (Oliach et al., 2021), being truffle production a profitable investment in Mediterranean countries, especially in Spain.

Stakeholders identified the insufficient accessibility to irrigation at a reasonable cost as the main barrier for truffle production. Proper irrigation plays an important role in the success of truffle growing (Büntgen et al., 2015) while water resources are becoming scarcer due to climate change (Van De Loo et al., 2024). In this context, agroforestry systems could significantly increase rainfall locally in desert regions, but such systems may result sustainable only if the rainfall amount is higher than the amount of water consumed by the planted trees (Wang & D'Odorico, 2019).

The main reason chosen by the stakeholders for implementing intercropping in truffle orchards was obtaining an extra income from the secondary crop. Diversifying the production and market outlets is a strategy often adopted by small farmers to reduce the risk related to output price, yield and inputs (De Roest et al., 2018). In the case of truffle production, diversification could be beneficial in a two–fold way: the production of a secondary crop could reduce the price risks, particularly if the risks attached to the different crops are unrelated (McNamara & Weiss, 2005; De Roest et al., 2018), and it could result in soil quality enhancement and natural enemies' attraction thereby improving the truffle cultivation conditions, the latter scored as the second more important reason for stakeholders to implement intercropping systems. The importance of this incentive could be linked with the high prominence attached to biodiversity and soil enhancement services by the stakeholders, but without exceeding the importance attached to the truffle production service.

Although it may appear that the stakeholders leaned mostly towards the productive dimension of the truffle–producing systems, the cluster analysis revealed that these respondents in cluster 2, for whom truffle production was the most important ES, also had a prevailing ecocentric worldview. Forest ownership literature reveals that, far from being contradictory, forest owners strongly motivated by economic consideration may be as well highly concerned about other values related to forest ownership, being environmental values amongst them (Wiersum et al., 2005; Ní Dhubháin et al., 2007; Howley, 2013). Furthermore, this may as well reflect a holistic perspective of the human relationship with the environment, in which there is no clear line between utilitarianism and biocentrism, but rather a synergistic orientation more attached to the local social and cultural values (Van Petegem & Blieck, 2006; Ogunbode, 2013).

4.2. Wildfire protection and other regulating services

Following truffle production, wildfire protection linked to truffle–producing systems was valuated as the most important among the regulating services. Wildfire–resilient landscape outcomes could be produced by combining direct and indirect actions (Wunder et al., 2021; Colonico et al., 2022). While direct measures are actions aimed at implementing strategic fuel management actions (e.g., fuel breaks), indirect measures encompass supporting rural and natural land development, such

as sustainable forest management (Verkerk et al., 2018) or agro–pastoral activities (Moreira & Pe'er, 2018; Varela et al., 2020), which have as side–effects the reduction of fire–hazard and the improvement of the landscape fire regulation capacity. Previous studies conducted within the Mediterranean region have shown that the active management of abandoned farmlands in mountain landscapes pays–off in terms of increased resilience to wildfires (Sil et al., 2019; Pulido et al., 2023; Spadoni et al., 2023), and the issue raised by these authors is how to make these land management systems profitable. In this framework, truffle cultivation may provide a sound, financially viable alternative to contribute to wildfire regulation in these abandoned rural landscapes that are more vulnerable to wildfires.

Beyond wildfire protection, further regulating services with high importance for stakeholders were the biodiversity and soil health maintenance, and CO_2 sequestration enhancement. Indeed, agroforestry systems are considered valuable landscapes involving a sustainable land management strategy that can improve a number of common indicators that define biodiversity and soil health (Dollinger & Jose, 2018; Elevitch et al., 2018).

4.3. Cultural dimension

Agroforestry systems are also known for supporting rural cultural heritage (Torralba et al., 2018). In the case of truffle–producing systems this ES together with aesthetic enjoyment of truffle landscapes were, overall, the least important for stakeholders. However, the cluster analysis allowed to reveal that cultural heritage was the most important ES for a group of stakeholders. Cultural heritage refers to these biophysical features, physical artifacts, and intangible characteristics of a group or society that present generation inherited from the past and transfer them to future generations (Czepczyński, 2016). Both tangible and intangible attributes are related to cultural heritage as ES (e.g., the practices of truffle hunting and the tools employed for it), including the landscape configuration and specific tree species (e.g., holm oaks) (Daniel et al., 2012). Cultural landscapes contribute to the identity of communities (Stephenson, 2008) and, as exemplified in truffle production systems, culture is changeable and often induces ecosystem changes (Van Eetvelde & Antrop, 2009). Thus, preserving cultural heritage can have considerable synergy with preserving other ES. The cultural aspect of truffle–growing ecosystems has been underlined (Therville et al., 2013), and it can constitute a key value since cultural heritage services can be directly experienced and intuitively appreciated by consumers. Linked to this cultural dimension, truffles can be conceptualised as landscape products, embodying the relationships between the multifunctionality of food and the sustainability of agricultural landscapes (García-Martín et al., 2021). The cultural dimensions of truffle cultivation are associated with the pride of truffle cultivators, as well as their appreciation by distant

consumers and may constitute a source of income diversification for the landowners by marketing some of these services for tourists.

4.4. Suitability of New Environmental Paradigm analysis to measure ecological overviews

In this study we adopted the NEP scale to measure environmental attitudes. The universal nature of the beliefs measured by NEP may explain why it has become the most widely used measure of environmental attitudes since its publication in 1978 (Hawcroft & Milfont, 2010). The NEP is situated at the opposite side of consumerism, individualism and the attitude with anti–ecological values that often characterise human relationship with the environment (Dunlap, 2008). This relationship leaves societies with two solutions in order to regulate human activity: either to fit into the world harmoniously by trying to preserve it (harmony values, or the NEP worldview), or to exploit and change it (mastery values, or the dominant social paradigm worldview) (Hawcroft & Milfont, 2010). However, the social attitudes from which some environmental problems derive may not always reflect similar psychosocial structures as represented in the NEP scale (Ogunbode, 2013). Alternatively, culture and direct experiences of severe environmental problems (Pierce, 1997; Vikan et al., 2007) do also shape environmental attitudes and beliefs, and citizens of traditional societies have been found to concurrently hold ecological and utilitarian disposition towards environmental issues (Bechtel et al., 1999; Corral-Verdugo & Armendáriz, 2000).

Our sampled individuals may well showcase these apparent contradictions in applying the NEP to respondents that work closely in the conservation of traditional cultural landscapes. Cultural heritage is inextricably linked to historical relationships between human societies and ecosystems. Cultural landscapes are significant constituents of cultural heritage characterised by the long–term interaction between site conditions and human practices. Therefore, we postulate that the stakeholders in cluster 3 reject the more pro–environmental dimensions in the NEP since these relate to a conception of human beings as potentially being detrimental to nature that collides with their beliefs and values. Future assessment of environmental worldviews in truffle–producing systems may well explore their perceptions in open–ended formats to explore the interplay of environmental and cultural values hold by these actors.

4.5. Policy and/or managerial implications

Our results suggested a plurality of values leading to a high potential for landscape multifunctionality that may give, in the future, way to trade–offs among different stakeholder groups (Plieninger et al., 2023). A strategic spatial distribution of agro–forestry and agricultural land use patches may contribute to reduce overall landscape biomass content, and hence fire intensity,

improving the opportunities for fire extinction while eventually reducing suppression costs (Lecina-Diaz et al., 2023; Pulido et al., 2023). Therefore, providing wildfire protection services, especially in Mediterranean truffle-producing systems, can be considered in policies, acknowledging the multifunctional role of these systems beyond mere production. At the same time, given the considerable irrigation cost of truffle plantations, the need for research and implementation of new, water– and cost–efficient techniques in truffle orchards is risen. In addition, the income diversification can be supported by the implementation of intercropping systems in the early stages of truffle plantations, which will allow innovation to thrive through cultural heritage. For this, a multi–sector collaboration is needed to avail the multiple functions of truffle-producing systems. Finally, the shared appreciation of truffles as landscape products should be reinforced in food and biodiversity policies in order to support natural conservation and sustainable development of these landscapes (García-Martín et al., 2022). In this sense, truffle–producing systems should be considered as multifunctional systems, providing multiple services to society beyond truffle, and this view has been backed up by the local actors, as shown in our study.

5. Conclusions

In this study, the ES associated to truffle-producing systems were evaluated by directly implicated stakeholders. While the provisioning service of truffle production was evaluated as the most important, the regulating services of wildfire protection, biodiversity and soil health maintenance, and CO_2 sequestration enhancement, were also highly ranked. The cultural ES of cultural heritage and landscape enjoyment were considered very important for a specific group of stakeholders, revealing the multifunctional aspect of these ecosystems.

Among the barriers related to the truffle sector, stakeholders pointed out an applied problem, the irrigation of truffle plantations at a reasonable cost, implying potential policy needs that would promote the sustainability of these ecosystems. Moreover, given the long time to achieve a return on the invested capital on truffle orchards, truffle growers would be motivated to implement of a secondary crop during the first years, provided the additional income and the ecological side benefits derived from the secondary crop.

Decision–making should take into consideration the environmental value of truffle-producing systems, as well as their social significance support the sector holistically. In this context, our findings can be used by local governments and/or academia, to enhance further ES assessments associated with truffle-producing ecosystems.

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7. Supplementary material

Figure S1 Example of comparative importance between two ecosystems services (ES) assessed by Analytic Hierarchy Process. The starting point is that the two ES are equally important, and the respondents were asked to evaluate whether, and how much, one of them is more important than the other.

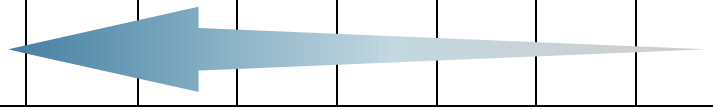
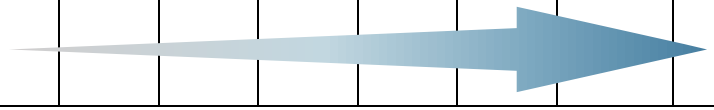
Truffle production								***vs***	**Soil quality maintenance**							
Absolutely more important		Much more important		More important		A bit more important		Equally important	A bit more important		More important		Much more important		Absolutely more important	
9	8	7	6	5	4	3	2	1	2	3	4	5	6	7	8	9
																

Table S1 The statements of the 15-item New Environmental Paradigm (NEP) used to evaluate the environmental attitudes of respondents.

NEP	Statement
NEP_1	We are approaching the limit of the number of people the Earth can support.
NEP_2	Humans have the right to modify the natural environment to suit their needs.
NEP_3	When humans interfere with nature it often produces disastrous consequences.
NEP_4	Human ingenuity will ensure that we do not make the Earth unliveable.
NEP_5	Humans are seriously abusing the environment.
NEP_6	The Earth has plenty of natural resources if we just learn how to develop them.
NEP_7	Plants and animals have as much right as humans to exist.
NEP_8	The balance of nature is strong enough to cope with the impacts of modern industrial nations.
NEP_9	Despite our special abilities, humans are still subject to the laws of nature.
NEP_10	The so-called "ecological crisis" facing humankind has been greatly exaggerated.
NEP_11	The Earth is like a spaceship with very limited room and resources.
NEP_12	Humans were meant to rule over the rest of nature.
NEP_13	The balance of nature is very delicate and easily upset.
NEP_14	Humans will eventually learn enough about how nature works to be able to control it.
NEP_15	If things continue on their present course, we will soon experience a major ecological catastrophe.

Table S2 Demographic characteristics of the sample (*N* = 135).

Demographics		***N***	**Frequency (%)**
Age	18-40	38	28.15
	40-65	87	64.44
	>65	10	7.41
Gender	Male	97	71.85
	Female	37	27.41
	Prefer not to say	1	0.74
Truffle plantation owner	Yes	105	77.78
	No	30	22.22
Truffle plantation owner and professional as:	Researcher / technician	16	15.24
	Truffle hunter	23	21.90
	Nursery farmer	0	0.00
	Worker in restaurant / truffle-processing company	3	2.86
	None of the above	63	60.00
Not truffle plantation owner but, professional as:	Researcher / technician	19	63.33
	Truffle hunter	5	16.66
	Nursery farmer	1	3.33
	Worker in restaurant / truffle-processing company	1	3.33
	Other	4	13.33

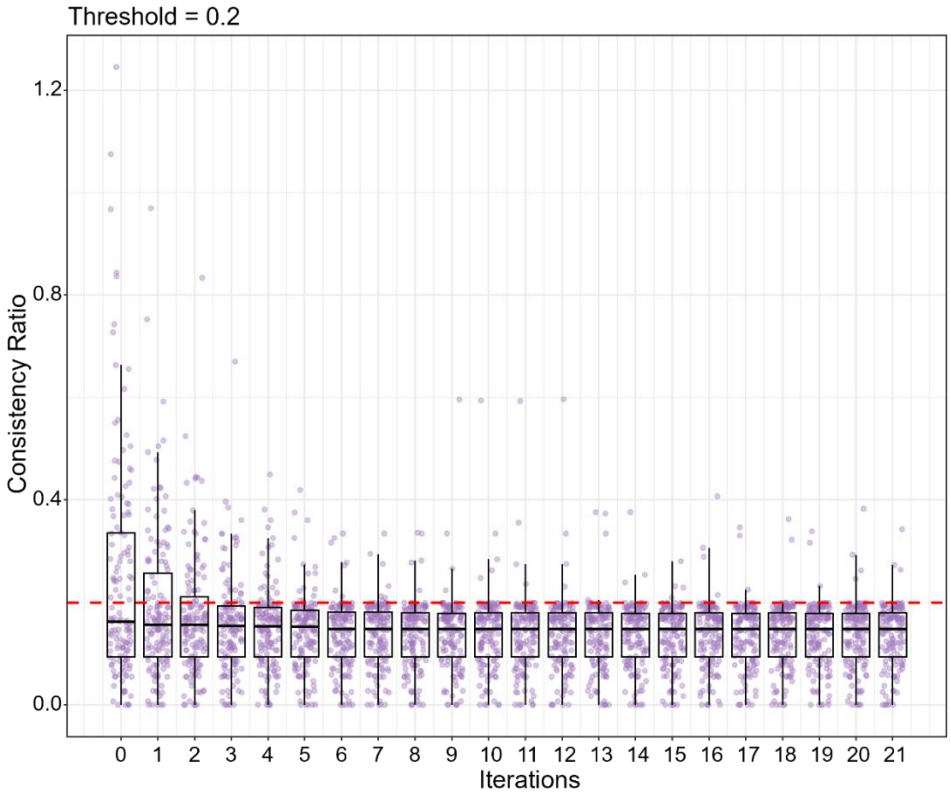


Figure S2 AHP consistency ratio showing the iterations needed in order to reduce inconsistency (threshold = 0.2).

Text S1 Analysis of the 15-item New Environmental Paradigm (NEP).

The 15-item NEP was first analysed and Bartlett's test of sphericity (Chi-square$_{105}$ = 387.01, $p < 0.001$) and Kaiser-Meyer-Olkin test (KMO = 0.76) suggested that the data were appropriate for factor analysis. The Cronbach's alpha ($\alpha = 0.76$) showed that the 15-item NEP had a satisfactory level of internal consistency and therefore, were reliable. We retained the first five principal components (PCs), considering eigenvalues higher than 1 (**Table S3**) and accounting for more than 60% of the explained variance, as selection criteria for varimax rotation. By checking the rotated loadings, statements NEP 10 and NEP 11 showed values lower than 0.50 in all factor loadings while the communality value of NEP 3 was lower than 0.50 (**Table S4**, **Table S5**), thus these three NEP items did not meet the minimal requirements for analysis and were excluded from the dataset.

Table S3 Principal components analysis with the 15-item of New Environmental Paradigm (NEP).

Component	Initial Eigenvalues		
	Eigenvalue	% of Variance	Cumulative %
1	3.898	25.99	25.99
2	2.048	13.65	39.64
3	1.139	7.59	47.24
4	1.092	7.28	54.52
5	1.053	7.09	61.54
6	0.956	6.38	67.91
7	0.821	5.48	73.39
8	0.690	4.60	77.99
9	0.629	4.19	82.18
10	0.570	3.80	85.98
11	0.554	3.70	89.68
12	0.460	3.07	92.75
13	0.404	2.69	95.44
14	0.369	2.46	97.90
15	0.315	2.10	100

Table S4 Varimax rotation loadings and communality values of the 15-item of New Environmental Paradigm (NEP).

NEP item	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Communality value
NEP_1	0.51	-0.198	0.275	0.081	-0.349	0.5
NEP_2	-0.101	-0.073	-0.131	0.896	0.131	0.85
NEP_3	0.524	-0.107	0.204	-0.237	0.259	0.45
NEP_4	0.005	0.018	-0.221	0.159	0.808	0.73
NEP_5	0.807	-0.122	0.116	-0.163	-0.076	0.71
NEP_6	0.211	0.73	-0.208	-0.193	0.137	0.68
NEP_7	0.639	-0.055	0.234	-0.047	0.203	0.51
NEP_8	-0.082	0.311	-0.683	0.111	0.017	0.58
NEP_9	0.699	0.253	0.137	0.158	-0.172	0.63
NEP_10	-0.333	0.419	-0.436	0.086	0.194	0.52
NEP_11	0.456	0.418	0.216	0.366	-0.043	0.57
NEP_12	-0.371	0.649	-0.086	0.087	0.041	0.57
NEP_13	0.209	-0.053	0.72	-0.018	0.032	0.57
NEP_14	0.013	0.471	0.249	-0.028	0.622	0.67
NEP_15	0.29	0.155	0.749	-0.008	-0.14	0.69

Table S5 Principal components analysis with the questions of the 12-item of New Environmental Paradigm (NEP).

Component	Initial Eigenvalues		
	Eigenvalue	% of Variance	Cumulative %
1	3.257	27.15	27.15
2	1.777	14.81	41.96
3	1.114	9.28	51.24
4	1.086	9.05	60.29
5	0.968	8.06	68.35
6	0.815	6.79	75.14
7	0.687	5.73	80.87
8	0.618	5.15	86.02
9	0.506	4.21	90.23
10	0.461	3.85	94.08
11	0.38	3.16	97.24
12	0.331	2.76	100

Table S6 Communality of the 12-item of New Environmental Paradigm (NEP).

NEP item	Communality value
NEP_1	0.69
NEP_2	0.85
NEP_4	0.72
NEP_5	0.75
NEP_6	0.73
NEP_7	0.61
NEP_8	0.7
NEP_9	0.59
NEP_12	0.52
NEP_13	0.6
NEP_14	0.69
NEP_15	0.75

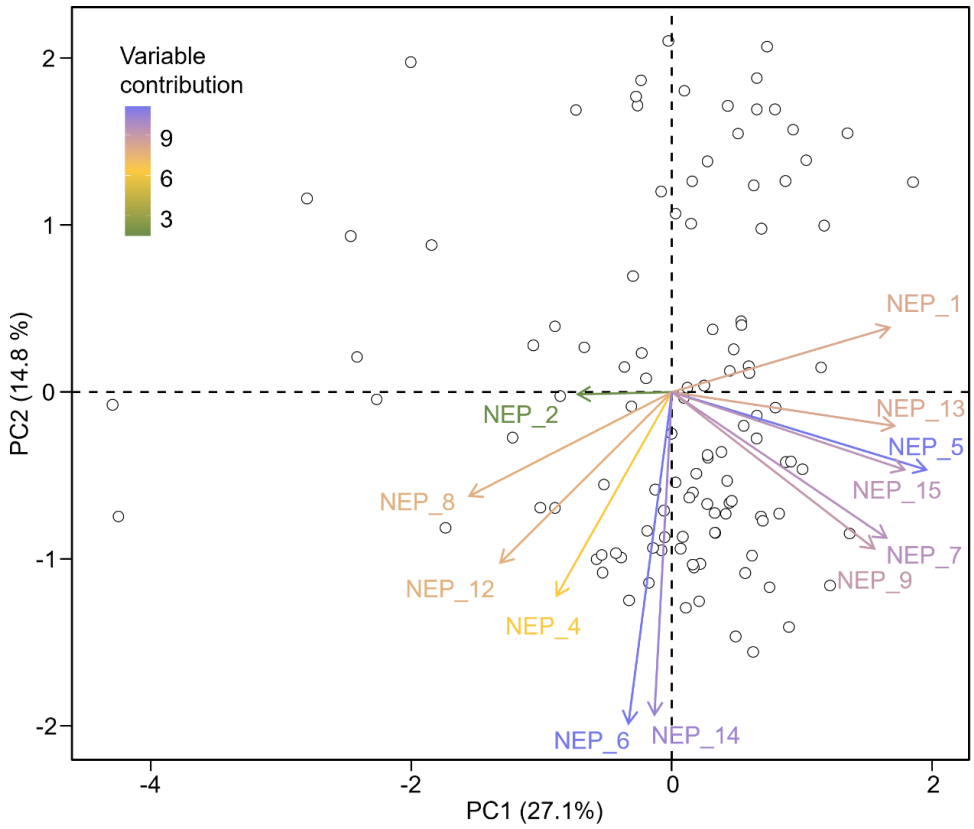


Figure S3 The first two axes of the principal components analysis (PCA) with varimax rotation over the 12-item of New Environmental Paradigm (NEP) (see **Table S1**).

Appendix

1. What are the pros and cons of truffle-producing forests, truffle orchards and intercropping truffle orchards (e.g. combination of truffle-inoculated trees and aromatic plants, vineyard, legumes or cereals) from an environmental, economic and social point of view?

2. In case of shifting from a monoculture truffle-orchard to intercropping, how important the following factors would be to encourage/discourage this shifting, rating from 1-not important at all, to 5-very important?

Factor
The adequacy of climate and soil for the secondary crop production.
The establishment/extension of the irrigation system for the secondary crop.
The availability of extra manpower needed for the secondary crop production.
The field capacity of the truffle plantation for the secondary crop.
The experience of truffle grower in the cultivation of the secondary crop.
The availability of machinery to harvest the secondary crop.
The availability of adequate machinery to process the harvested crops.
The space available to store the harvested crops.
The available market to sell the products of the secondary crop.
The incentives (e.g. subsidies) for truffle growers to implement intercropping by the local authorities.

3. We identified the main stakeholder groups related to truffle productive systems: truffle farmers, wild truffle collectors, truffle specialised researchers or technicians working in the local administration, owners of truffle nursery and employees who prepare/sale products made with truffle in restaurants or companies, locals and visitors. Putting yourself in the place of the previously identified groups, what advantages and disadvantages would you perceive in relation to the three types of truffle-producing systems (i.e. forest, plantation, intercropping system)?

4. The following ecosystem services (ES) have been classified by CICES (Common International Classification of Ecosystem Services) and could be provided by any type of the truffle-producing systems.

   (a) Is the description of the ES clear or could get better? Do you miss any ES?
   (b) Please evaluate the capacity of each truffle-producing system to provide the following ES, from 1-no capacity to provide the ES, to 5-a lot of capacity to provide the ES.
   (c) Which ES would you consider as the five most important for you and for each one of the stakeholder groups?

Ecosystem services (ES) that truffle-producing systems can potentially provide				
Type of service	**ES**	**Description**	**Example**	**Measurement**
Provision	**P1.** Truffle production in agricultural systems.	The primary service of these systems is to produce the fruit of *Tuber melanosporum* and other *Tuber spp.*	Truffle production in oak trees.	Kg. of truffle produced annually per hectare.
	P2. Production of aromatic plants or other secondary crops in intercropping systems.	Grow aromatic plants (or other crops) while waiting for the truffle plantation to start producing truffles.	Intercropping truffle oaks with lavender or other crops.	Litres of essential oil produced/gr. of flowers per hectare.
	P3. Animal production.	Use oak groves (forests/plantations) as pastureland for livestock, which feed on grass and acorns.	Goats, sheep, and cows grazing in oak woodlands.	Kg. of meat from animals grazing in oak woodlands.
	P4. Wild truffle production.	Harvesting truffles in the wild.		Kg. of wild truffe produced per hectare.
	P5. Mushroom and edible wild plants production.	Collection of wild mushrooms and edible plants in natural oak forests.	Mushroom park	Kg. of harvested mushrooms/edible plants.
	P6. Firewood production.	Using firewood from truffle-producing oak trees as a source of energy.	Silviculture	Firewood volume (m^3/hectare).
	P7. Truffle inoculum production in forests.	Wild truffles can be used to inoculate oak trees for new truffle plantations.	Traceability of truffle origin.	Potential number of inoculated plants.
	P8. Acorn producción as propagating material.	Acorns from the forests can be used to create oak seedlings better adapted to each area.	Traceability of plant origin.	Kg. of acorns de bellotas recollected per hectare as propagating material.
Regulation and maintenance	**R1.** Soil erosion control.	Oak trees help prevent soil erosion.	Oak groves beneficial for soil in extensive crop areas (e.g., cereal crops).	Tonnes of soil retained by an oak forest compared to bare soil or other land uses.
	R2. Maintenance of hydrological cycle.	Trees, with their roots, help retain water in the soil.		Soil water potential, with and without vegetation cover.
	R3. Fire risk reduction.	The structure of truffle oak forest reduces fire spread compared to denser forests with a ladder-like forest fuel structure.	Mosaic landscape.	Hectares of forest with a fuel model of low fire propagation.
	R4. Promotion/maintenance of pollinator diversity.	The truffle-producing systems provide shelter to different pollinators that pollinate plants both in the truffle system and in adjacent crops and habitats.	Flowering crops, interspersed with truffle orchards.	Number and diversity of pollinators.
	R5. Provision of habitats and maintenance of biodiversity.	Truffle ecosystems provide the necessary conditions for the development of different species populations (biodiversity)	Insects, birds, micromammals, rodents, etc.	Number and diversity of plant and animal species (biodiversity).

	R6. Pest control in holm oaks and truffles.	Truffle ecosystems can provide a habitat for natural predators of pests that harm oak trees and truffles.		Abundance and density of species that are natural enemies of pests.
	R7. Control of holm oak diseases.	Truffle ecosystems can provide a home for natural predators of insects that spread oak tree diseases.		Abundance and density of species that are natural enemies of insects.
	R8. Soil quality maintenance (fertility, structure, health).	Oak trees and microorganisms contribute to improving soil quality.	Improve soil quality in terms of fertility, structure, health, and microbial biodiversity.	Structure and composition of the soil (organic matter, quality, and type of aggregates).
	R9. Carbon sequestration in soil and trees.	Ability of truffle systems to capture and store carbon in the soil.		Tonnes of sequestered CO_2.
	R10. Regulation of temperature and humidity.	Oak trees in these systems help regulate humidity and temperature, creating suitable conditions for other species. [Drawback: allelopathic effect of the truffle]		Number of species establishing under the canopy of oak trees compared to open areas without trees.
Cultural	**C1.** Offer space for recreational activities: hiking.	Truffle systems offer opportunities for hiking.		Number of visitors who practice hiking activities in these systems.
	C2. Offer space for recreational activities: observation of animals and plants.	Truffle systems offer opportunities for recreational activities such as wildlife and plant observation.		Number of visitors who practice nature observation activities in these systems.
	C3. Characteristics of truffle systems that stimulate scientific research.	Truffle systems provide opportunities for research to improve production.	Mushroom parks.	Number of scientific articles/projects related to truffles.
	C4. Characteristics of truffle systems as part of the cultural heritage or historical knowledge.	The practices of truffle cultivation, hunting, and harvesting are considered intangible cultural heritage.		Number of unique cultural practices, vocabulary, and tools linked to truffles.
	C5. Maintenance of oak groves/truffle plantations for the enjoyment of future generations.	The intangible values of truffle ecosystems motivate current owners to preserve their legacy.		Number of private owners who carry out management practices that will have a result beyond their generation.

General conclusions

Chapter 1

1. The black truffle mycelium shows a seasonal variation pattern probably connected to local soil-climatic factors, while the type of production (i.e., plantation or forest) does not affect the amount of mycelium biomass.
2. At the regional scale, the black truffle mycelium biomass is consistently influenced by a combination of climatic and soil factors, depending on the production type. Longitude, calcium carbonate, sodium and zinc are related, either positively or negatively, to soil mycelium amount in forests, while in plantations, the main related variables are the elevation, organic matter, phosphorus, magnesium and boron contents in soil.

Chapter 2

3. The abundance of black truffle, estimated by high-throughput sequencing, negatively impacts soil enzymatic activity in both natural and managed producing systems, alongside other specific biotic and abiotic soil variables differentially impacting in each producing system.
4. In truffle producing forests, soil enzymatic activity related to phosphorous cycling and recalcitrant carbon degradation is generally lower than in plantations, while nitrogen mobilisation is higher in forests than in plantations.
5. Independently of the truffle producing system, higher soil enzymatic activity related to rapid carbon and nitrogen turnover is observed in late spring compared to autumn which, given the strong site effect, could be explained by the local climatic conditions rather than by seasonal *T. melanosporum* dynamics.

Chapter 3

6. Black truffle productive plantations exhibit more homogeneous soil fungal communities and complex fungal co-occurrence network compared to forests, likely indicating more stable fungal communities in relation to the uniform environmental conditions of monoculture plantations.
7. *Tuber melanosporum* does not act as keystone species structuring the soil fungal network within the brûlé in any truffle-producing system, even though it likely has a more central role in forests than in plantations.

8. Despite the strong local site effect, regional patterns concerning nitrogen cycling in soil mainly related with saprotrophs have been revealed, but only in plantations and not in forests.

Chapter 4

9. The co-culture of truffle-oaks and medicinal and aromatic plants significantly influences the growth and development of plant and soil fungal symbionts in mesocosm conditions.

10. The presence of medicinal and aromatic plants negatively impacts the growth of truffle-oaks and *T. melanosporum* abundance, especially when they are inoculated with native arbuscular mycorrhizal fungi that likely compete with *T. melanosporum* for colonisation of truffle-oak's roots.

11. Medicinal and aromatic plants growth is mostly unaffected by the co-culture with truffle-oaks, while the inoculation with native arbuscular mycorrhizal fungi is beneficial for their growth, especially for lavender.

Chapter 5

12. Truffle production is considered by stakeholders as the most important provisioning service provided by truffle ecosystems. The regulating services attached to these ecosystems are also recognised as significant for biodiversity and soil conservation, while cultural services are particularly important for stakeholders showing landscape awareness.

13. Irrigation at a reasonable cost is recognised by most stakeholders as a significant barrier for truffle cultivation sustainability.

14. Truffle growers are interested in implementing secondary crops to generate additional income and ecological benefits during the initial years of truffle orchard establishment.

Black truffle (*Tuber melanosporum* Vittad.) interactions with soil and plants in productive systems, and associated ecosystem services

Phd Dissertation

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2024